universität wien

DISSERTATION

Titel der Dissertation

Analysis of self-renewal in developing mouse brain

Verfasser

Marko Repic

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 091490
Dissertationsgebiet lt. Studienblatt:	Dr.-Studium der Naturwissenschaften Molekulare Biologie UniStG
Betreuerin / Betreuer:	Dr. Jürgen Knoblich

TABLE OF CONTENTS

ABSTRACT

During neurogenesis in the mammalian neocortex, neural progenitors termed radial glia undergo symmetric and asymmetric cell divisions to self-renew and produce differentiating neuronal progeny. However, how choices between differentiation and self-renewal are regulated is not fully understood yet.

We use transcriptome analysis of FACS-sorted cortical radial glia and neurons combined with *in vivo* RNAi screening to identify novel factors regulating neurogenesis in the embryonic neocortex. We unravel a novel function of AU-rich element-mediated mRNA decay in regulation of self-renewal of cortical radial glia. Tis11b protein recruits mRNA decay machinery to the specific AU-rich mRNAs. It is highly expressed in radial glia but low in differentiated neurons. RNAi-mediated depletion of Tis11b in embryonic neocortex impairs self-renewal capacity of the radial glia, leading to the premature neuronal differentiation. We identify two genes that promote neuronal cell fate, Neurogenin2 and Delta-like 1, as novel targets of Tis11b. We further demonstrate that expression of Tis11b is positively controlled by Notch signalling. Thus, we establish a new link between Notch pathway and turnover of mRNAs containing AU-rich elements.

Based on our results, we propose that Tis11b prevents premature exhaustion of progenitor pool by promoting mRNA decay of genes that induce neuronal differentiation.

In the second part of the thesis, we show an essential role for NKAP (NF-kappaB activating protein) in cortical development. Conditional deletion of NKAP gene with Emx1-cre in dorsal telencephalon leads to massive apoptosis of neural progenitors around the onset of neurogenesis, resulting in complete loss of entire cerebral cortex by the end of prenatal development. Our results reveal a critical role for NKAP in survival of neural progenitors in the embryonic neocortex.

ZUSAMMENFASSUNG

In der Neurogenese des Saeugetierneokortex teilen sich neuronale Vorlaeuferzellen, Radialgliazellen entweder symmetrisch oder asymmetrisch um sich einerseits selbst zu erhalten und andererseits ausdifferenzierende Tochterzellen zu bilden. Die Frage, wie die Wahl zwischen Selbsterhaltung und Ausdifferenzierung getroffen wird, ist jedoch noch weitgehend unbeantwortet.

Wir benutzen Transkriptomdaten von FACS-gereinigten Radialglia und Neuronen in Zusammenspiel mit *in vivo* RNA-Interferenz, um neue regulatorische Faktoren der Neurogenese im embryonalen Neokortex zu identifizieren. Wir zeigen eine neue Funktion fuer den mRNA Abbau AU reicher Elemente in der Selbsterhaltung kortikaler Radialglia. Das Protein Tis11b, welches mRNA Abbau vermittelnde Proteine an bestimmte AU reiche mRNA Sequenzen rekrutiert, ist stark in Radialglia, hingegen nur schwach in Neuronen exprimiert. RNA-Interferenz induzierter Verlust von Tis11b in Radialglia fuehrt zu verringerter Selbsterhaltung und erhoehter neuronaler Differenzierung. Weiters identifizieren wir zwei neuronale Differenzierungsgene, Neurogenin 2 und Delta-like 1, die durch Tis11b gesteuert werden. Tis11b ist seinerseits abhaengig von Notch Signalen. Entsprechend zeigen wir eine neue Verbindung zwischen Notch Signaltransduktion und dem mRNA Abbau AU reicher Elemente. Unsere Daten zeigen, dass Tis11b die vorzeitige Differenzierung von Radialglia hemmt, indem es den Abbau der mRNA von Differenzierungsfaktoren induziert.

Im zweiten Teil dieser Arbeit zeigen wir eine essentielle Funktion fuer das Gen NKAP (NF-kappaB activating protein) in der kortikalen Entwicklung. Verlust von NKAP im dorsalen Telenzephalon fuehrt zu weitgehendem Zelltod neuronaler Vorlaeuferzellen am Beginn der Neurogenese, welches in dessen Folge zum kompletten Verlust des celebraren Kortex fuehrt. Entsprechend zeigen unsere Daten eine kritische Rolle fuer NKAP als Ueberlebensfaktor im embryonalen Neokortex.

INTRODUCTION

Brain is undoubtedly the most complicated organ in the mammalian body. During mammalian embryonic development, single layer of neuroepithelial cells forming the neural tube serves as a precursor for the entire central nervous system. Neural progenitors in the dorsal part of the forebrain will produce the majority of the neurons in the neocortex, which is the centre of the higher brain functions. Tightly regulated control of progenitor proliferation and differentiation is essential for the development of anatomically and physiologically normal brain. Defects in neurogenesis can result in severe developmental disorders such as microcephaly. Therefore, detailed understanding of mechanisms that control the balance between self-renewal and differentiation is of particular interest.

Organisation of the embryonic forebrain

The embryonic forebrain can be divided into two major parts the dorsal telencephalon (pallium) and the ventral telencephalon (subpallium) (Guillemot, 2005) (Fig. 1).

The dorsal telencephalon can be subdivided mediolaterally into choroid plexus, cortical hem, medial pallium, dorsal pallium and lateral pallium. Choroid plexus functions as a producer of cerebrospinal fluid. Cortical hem is a transient structure of the embryonic cortex located between choroid plexus and prospective hippocampus, where majority of the Cajal-Retzius neurons are born (Garcia-Moreno et al., 2007; Yoshida et al., 2006). Medial pallium will become the future hippocampus. Dorsal pallium will give rise to the six-layered neocortex. Progenitors of the lateral and ventral pallium will contribute to the formation of the olfactory cortex and nuclei of the amygdalar complex (Ceci et al., 2012; Georgala et al., 2011).

In the ventral telencephalon, the ganglionic eminences are the primary sites for neurogenesis of inhibitory GABAergic neurons. Furthermore, the ventral telencephalon will form subcortical basal ganglia.

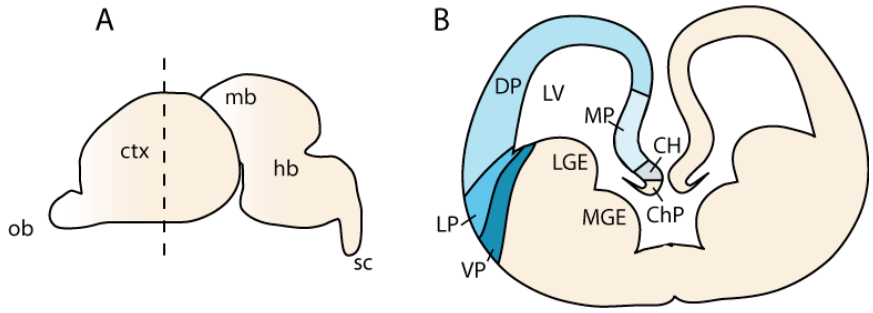


Figure 1. Anatomy of the developing brain. A. Lateral view of the mid-neurogenesis mouse brain. olfactory bulb (ob), cerebral cortex (ctx), midbrain (mb), hindbrain (hb), spinal cord (sc). Dashed line shows approximate position of the section shown in B. **B.** Coronal section of the embryonic forebrain. Choroid plexus (ChP), cortical hem (CH), medial pallium (MP), dorsal pallium (DP), lateral pallium (LP), ventral pallium (VP), lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE).

Progenitors in the developing neocortex

Neuroepithelium

Prior to the onset of neurogenesis, the neural tube is composed of the neuroepithelial cells, which will give rise to virtually all cells of the central nervous system. The neuroepithelial cells form a single layer of pseudostratified epithelium and exhibit typical features of epithelial cells such as apical-basal polarity and formation of tight and adherens junctions (reviewed in Gotz and Huttner, 2005). Core components of the polarity complex Par3 Par6 and aPKC (atypical protein kinase C) are localized in the apical process of the neuroepithelial cells facing the lumen of the neural tube (Manabe et al., 2002). The important role of the polarity complex in regulation of mammalian neurogenesis has been well described. It has been shown that polarity protein Par3 is important for cell fate decisions, since reduction of Par3 by RNAi results in increased neuronal differentiation (Bultje et al., 2009; Costa et al., 2008). Overexpression of Par3 or Par6 promotes proliferative divisions at the expense of neuronal production (Costa et al., 2008). Additionally Par3 was also suggested to act as a cell fate determinant that could segregate asymmetrically into one of the daughter cells during mitosis of the neural progenitors (Bultje et al., 2009). Par complex also plays an important role in *Drosophila* neurogenesis,

indicating that Par complex machinery is an evolutionary well-conserved mechanism to regulate cell fate decisions during the brain development (reviewed in Knoblich, 2008).

Another hallmark of the neuroepithelial cells is the interkinetic nuclear migration or the oriented movement of the nucleus along the apical-basal axis that is correlated with the cell cycle stage (Sauer, 1935). Neuroepithelial cells invariantly undergo mitosis at the apical surface of the neural tube. During G1-phase the nucleus moves to the basal side, where S-phase of the cell cycle occurs. This is followed by the migration of the nucleus in G2-phase back to the apical surface, where another cell division takes place (reviewed in Latasa et al., 2009).

Significance of the interkinetic nuclear migration is not well understood. It has been shown that the interkinetic nuclear migration is not required for cell cycle progression, since inhibition of myosin II, which is required for movement of the nucleus in basal direction, does not impair progression of the cell cycle (Schenk et al., 2009). On the other hand blocking the progression of the cell cycle interferes with the migration of the nucleus, indicating that interkinetic nuclear migration is tightly coupled to the progression of the cell cycle (Ueno et al., 2006). Work in zebrafish retinal progenitors has shown that during interkinetic nuclear migration, progenitor cells are exposed to different levels of Notch signalling (Del Bene et al., 2008). Authors of the study have proposed that exposure of the progenitors to the Notch gradient during the interkinetic nuclear migration influences decisions between proliferative versus neurogenic divisions.

Radial glial cells

At the onset of neurogenesis, around embryonic day 10 in mice, the neuroepithelial cells start exhibiting certain characteristics of the glial cells and transform into neural progenitors termed radial glia. Radial glial cells express a variety of astrocyte markers such as calcium binding protein (S100β), astrocyte specific glutamate transporter (GLAST) and brain lipid binding protein (BLBP) (reviewed in Gotz and Huttner, 2005). In some species, but not in mouse, radial glia express glial fibrillary acidic protein (GFAP) (Malatesta et al.,

2000). Radial glia can also be reliably identified by expression of transcription factors Pax6 and Sox2.

Analogously to the neuroepithelial cells, radial glial cells have bipolar morphology and their processes span the entire length of the cortex. Nuclei of the radial glial cells undergo interkinetic nuclear migration. However they do not migrate the entire length of the cortical thickness, but remain confined to the ventricular zone (VZ), an area proximal to the lumen, that houses cell bodies of the radial glia.

It has been described that during neural development, processes of the radial glial cells provide a scaffold for the newborn migrating neurons (Rakic, 1972) and later in development, they would transform into astrocytes (Culican et al., 1990). However, they were generally not considered to be neuronal precursors, since they express markers of glial lineage.

The first line of evidence showing that radial glia can act as neural progenitors came from FACS-sorting experiments with the transgenic mice that express GFP in radial glial cells from human GFAP promoter (Malatesta et al., 2000). The progeny of the sorted cells, grown *in vitro*, constituted mainly of pure neuronal or astroglial cells. In later stages of development, towards the end of neuron production, neurogenic potential of the FACS-sorted radial glia became limited and progenitors displayed bias towards astrocyte differentiation. Further lineage tracing experiments *in vivo* confirmed that neurons in the neocortex are produced by radial gila (Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001). Fate mapping using transgenic BLBP-Cre mice has demonstrated that radial glia serve as neural progenitors in the entire central nervous system (Anthony et al., 2004).

Neurogenic divisions of radial glia

Asymmetric cell divisions of radial glia located in the cortex can produce neurons via two different modes of neurogenic divisions (Fig.2). During direct neurogenesis, the radial glia self-renew and produce a committed daughter cell which directly differentiates into a neuron. The newborn neuron often migrates along the basal process of the mother radial glia to reach its final location in the cortical plate (Noctor et al., 2001).

Indirect neurogenic divisions give rise to a self-renewing cell, and to an intermediate progenitor cell. Intermediate progenitors lose both apical and basal attachments and gain multipolar morphology (Attardo et al., 2008). The intermediate progenitors can be distinguished from the radial glia by expression of the transcription factor Tbr2 and the non-coding RNA Svet1 (Englund et al., 2005). The cells committed to the intermediate progenitor fate migrate into in the subventricular zone, the area located basally from the ventricular zone and usually divide once into two neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). In approximately 10% of divisions two more intermediate progenitors are born, each of them producing two neurons at the end of their cell cycle (Attardo et al., 2008; Haubensak et al., 2004; Noctor et al., 2004).

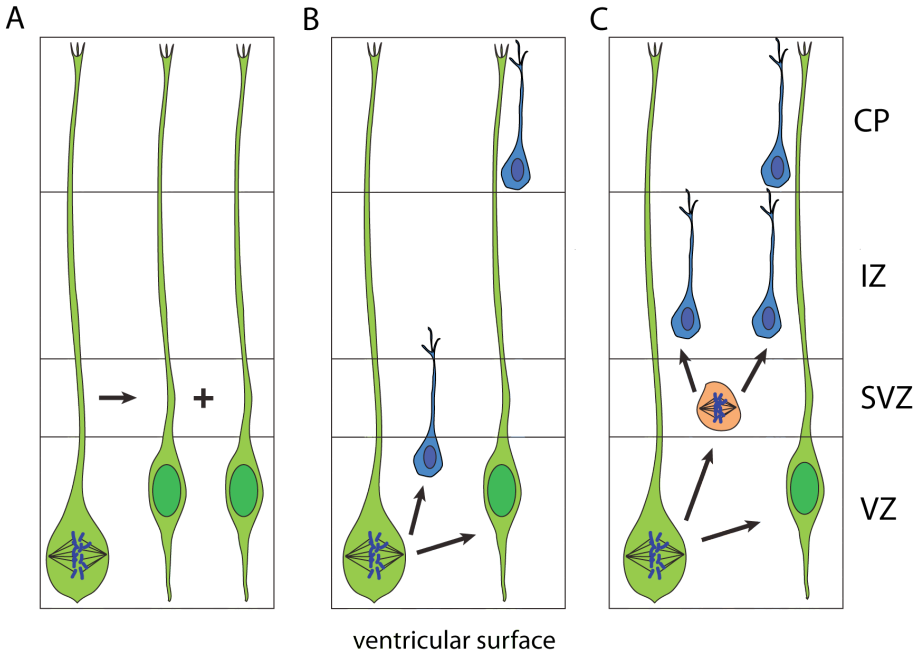


Figure 2. Division modes of radial glia. Radial glia (RG) undergo several types of divisions at the ventricular surface. **A.** Symmetric proliferative divisions expand the progenitor pool. Neurons can be generated via two different types of asymmetric division. **B.** Direct neurogenesis generates one self-renewing radial glia and one differentiating neuron. **C.** During indirect neurogenic divisions one cell self-renews to maintain RG identity, while other cell loses connection with the ventricular and pial surface to become an intermediate progenitor (IP). IP divide once more in the subventricular zone into two neurons. The newborn neurons migrate along the basal processes of the RG through the intermediate zone into the cortical plate. Ventricular zone (VZ), subventricular zone (SVZ) intermediate zone (IZ), cortical plate (CP). RG-green, IP-orange, neurons-blue.

The rate of direct versus indirect neurogenesis has been addressed by several studies. Analysis of the radial glial divisions around mid-neurogenesis at E13-14 estimated that 80% of the asymmetric divisions give rise to intermediate progenitors and the remaining 20 % to neurons (Miyata et al., 2004). Similarly, at the onset of neurogenesis the majority of neurogenic divisions are thought to produce an intermediate progenitor rather than a neuron (Attardo et al., 2008; Kowalczyk et al., 2009).

In contrast to direct neurogenesis, where one neuron per division of the radial glial cell is made, neurogenesis via intermediate progenitors produces two neurons per neurogenic division of the radial glial cell. Therefore intermediate progenitors represent a transit-amplifying step that expands neuronal output (Kriegstein and Alvarez-Buylla, 2009).

Mice with conditional deletion of the transcription factor Tbr2, which is the key determinant of intermediate progenitor fate, show a decreased forebrain size (Arnold et al., 2008; Sessa et al., 2008). Data from Arnold et al., 2008 shows that intermediate progenitors contribute to the production of upper-layer neurons. But Sessa et al., 2008 have observed decreased numbers of neurons in all neuronal layers of the Tbr2 cKO mice. This is consistent with the observations that intermediate progenitors contribute to neuronal production already at the onset of neurogenesis (Attardo et al., 2008; Haubensak et al., 2004; Kowalczyk et al., 2009). Additionally, mice lacking Insm1 (Insulinoma-associated 1) transcription factor, which is required for the generation of intermediate progenitor cells, have reduced numbers of neurons affecting all layers (Farkas et al., 2008). Thus, majority of the experiments show that intermediate progenitors contribute to neuronal production at all stages of cortical development.

Human patients with inactivated *EOMES* (*TBR2*) gene are born with microcephaly, resulting in mental retardation (Baala et al., 2007). Severe defects observed in these patients underline the importance of indirect neurogenesis in expansion of the cortical size and proper brain development.

Mechanisms that regulate decisions between direct and indirect neurogenesis are not well understood. Recently, a work from our lab has discovered the importance of mitotic spindle orientation during neurogenic divisions of radial glia (Postiglione et al., 2011). Mitotic spindles of the radial glia are predominantly positioned in parallel or oblique

orientation relative to the ventricular surface (Konno et al., 2008; Postiglione et al., 2011). Induction of vertical spindle orientation, via overexpression of the mitotic spindle regulator Inscuteable, increased fraction of progenitors undergoing indirect neurogenesis. Similarly, Inscuteable cKO mice have decreased numbers of intermediate progenitors and show lower rate of indirect neurogenesis.

Short neural precursors

Recently, another type of progenitors has been described in the ventricular zone of the embryonic neocortex. The short neural precursors express markers of the radial glia and have an apical process contacting the ventricular surface (Gal et al., 2006; Stancik et al., 2010). However, the basal process is variable in length and does not contact the pial surface (Gal et al., 2006). Stancik et al., 2010 proposed that compared to the radial glia, short neural precursors undergo just one or two mitoses and produce neurons directly.

Outer radial glia

In primates, another site of neurogenesis that is located in the outer subventricular zone contributes to the neuronal production during embryonic development. Neural progenitors of the outer subventricular zone have been recently characterised as outer radial glia (Hansen et al., 2010). Comparing to the radial glia in the ventricular zone, outer radial glia are in contact with the pial surface via basal process, but lack the apical process contacting the ventricle (Fietz et al., 2010; Hansen et al., 2010). It has been shown in humans that outer radial glia can undergo asymmetric divisions to self-renew and produce neurons (Hansen et al., 2010).

Rodent embryonic brains lack outer subventricular zone, nevertheless they contain a small number of outer radial glia-like cells (Shitamukai et al., 2011). Authors of the study have described that outer radial glia-like cells originate from the ventricular zone radial glia and their generation can be promoted by inducing oblique spindle orientation during mitosis of ventricular radial glia. The proposed role of spindle orientation in generation of outer radial glia-like cells is supported by data from the mice overexpressing Inscuteable, which have an ectopic layer of cells expressing radial glial markers outside of the ventricular zone

(Postiglione et al., 2011). Numbers of the outer radial glia are positively correlated with increase in the cortical size and their expansion likely represents an important step in evolution of the neocortex (Hansen et al., 2010; Lui et al., 2011; Shitamukai and Matsuzaki, 2012).

Development of 6-layered neocortex

Neurogenesis in mouse forebrain starts around day 10 of embryonic development (Kriegstein and Alvarez-Buylla, 2009). How asymmetric neurogenic divisions are initiated in the forebrain is not fully understood. However there is evidence for signalling from meninges regulating this process. For example, mice that carry a Foxc1 hypomorphic allele have a delay in the initiation of the neurogenic divisions (Siegenthaler et al., 2009). In these mutants, meninges covering the forebrain fail to develop properly. As a consequence, neuroepithelial cells lack all-trans retinoic acid (atRA), which is normally produced by meningeal cells. Treatment of the embryos with atRA can rescue the defects in neuronal production present in Foxc1 mutant mice (Siegenthaler et al., 2009). Another mechanism has been proposed for the rostral part of dorsal telencephalon, where FGF10 has been described to induce onset of neurogenesis (Sahara and O'Leary, 2009). FGF10 mutant mice show extended expansion of the neuroepithelial cells, followed by excessive neuron production resulting in increased cortical thickness in the rostral part of the dorsal telencephalon (Sahara and O'Leary, 2009).

During neurogenic period of cortical development, the neural progenitors in the dorsal cortex will generate excitatory pyramidal neurons that will be organized in six neuronal layers of the adult neocortex. Cortical excitatory neurons are the major neuronal type in the cerebral cortex. Around 25% of the cortical neurons fall into the category of inhibitory neurons that use GABA (γ-aminobutyric acid) as a neurotransmitter (reviewed in Wonders and Anderson, 2006). These GABAergic interneurons originate from progenitors in the ganglionic eminences of the ventral forebrain and migrate tangentially into the neocortex (reviewed in Hernandez-Miranda et al., 2010).

Different subtypes of cortical excitatory neurons are born in precise sequential order (reviewed in Molyneaux et al., 2007). Mechanisms controlling the sequential appearance

of different projection neuron subtypes are cell intrinsic. Several studies demonstrated that isolated cortical progenitors or embryonic stem cell-derived neural progenitors grown *in vitro*, produce different neuronal subtypes in sequential order that recapitulates the cortical development (Gaspard et al., 2008; Shen et al., 2006).

The first wave of newborn neurons migrates basally from the ventricular zone and forms a neuronal layer called preplate (Figure. 3). Cajal-Retzius cells are another type of neurons, which migrate into the preplate. These neurons are predominantly born outside of the neocortex in cortical hem and in pallial-subpallial border (Garcia-Moreno et al., 2007; Yoshida et al., 2006). Cajal-Retzius cells secrete Reelin molecule, which is essential for proper migration and positioning of the cortical projection neurons.

By the embryonic day 13, the second wave of newborn neurons splits the preplate into superficial marginal zone and deep subplate (reviewed in Gupta et al., 2002). Neurons of all subsequent layers migrate through the intermediate zone containing newborn neurons into the cortical plate. There, they adopt their final position below the marginal zone and on top of the previously born neuronal layer. The six-layered neocortex is formed in an inside-out fashion, where oldest neurons reside in the deep areas and younger neurons are located in more superficial cortical layers. Marginal zone becomes layer I of the adult brain. The subplate disintegrates after birth, and is not detectable in the adult brain anymore.

Neurons of individual layers differ in their morphology and connectivity. Neurons projecting into the thalamus are located in layer VI and neurons of the layer V mainly project to the subcortical areas, such as brainstem, cerebellum and spinal cord (reviewed in (Merot et al., 2009). Upper layer neurons (layer II-III) display abundant callosal projections (reviewed in (Molyneaux et al., 2007). Neurons of layers I and IV establish local connections within the hemisphere (reviewed in (Merot et al., 2009).

Around embryonic day E18 the first astrocytes are born in the cortex, marking the switch from neurogenesis towards gliogenesis (reviewed in Miller and Gauthier, 2007).

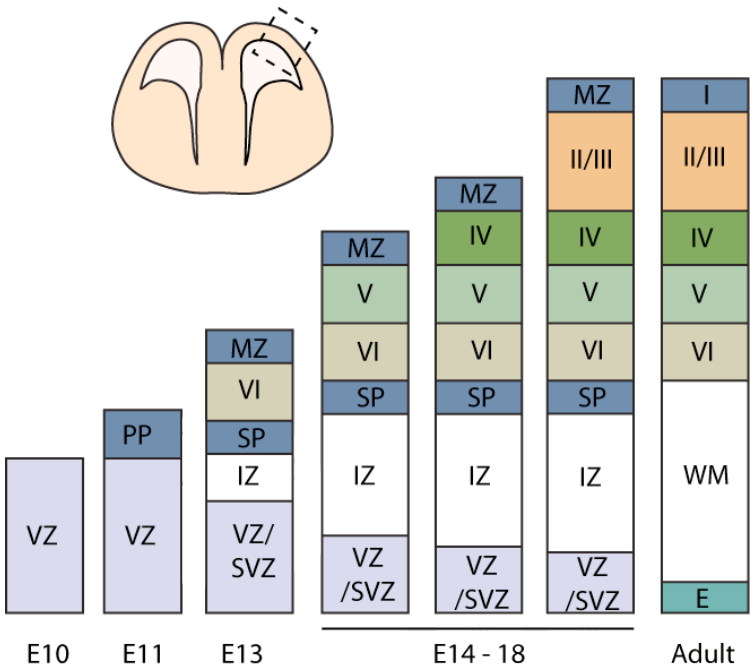


Figure 3. Development of six-layered neocortex during embryogenesis. The earliest-born neurons migrate basally from the ventricular zone forming a preplate. At E13, the next wave of newborn neurons splits preplate into marginal zone and subplate. Later born neuronal subtypes migrate on top of the previously formed layer, creating a six-layered neocortex in inside-out fashion. Subplate is a transient structure of the embryonic brain, which disappears after birth. Ventricular is reduced to the ependymal layer of the adult brain. ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), preplate (PP), subplate (SP), marginal zone (MZ), ependyma (E), white matter (WM).

Regulation of balance between self-renewal and differentiation

The successful cortical development resulting in normally functioning brain relies on intricate interplay between the factors that regulate proliferation and differentiation of neural progenitors. In larval brains of the fruit fly *Drosophila melanogaster*, the neural progenitors called neuroblasts, undergo invariant asymmetric divisions, producing one self-renewing neuroblast and one committed ganglion mother cell. The ganglion mother cell divides once more into two neurons (reviewed in Knoblich, 2008). Therefore the outcome of each cell division is predetermined.

However in mammalian neural tissue, decisions about the cell fate of the newborn cells are more plastic and do not necessarily follow the predetermined pattern. Lineage tree

analysis of cortical progenitors grown *in vitro* has shown that progenitors within one lineage can switch between the symmetric and asymmetric divisions (Shen et al., 2006). Mathematical analysis of the cell lineages supports the view that cell fate choices are not predetermined but rather contain a stochastic element in the cell fate decisions (Slater et al., 2009). Additionally, it has been observed in live cell imaging of cortical slice cultures of rat embryonic brains that individual radial glial cells can swith between indirect and direct neurogenic divisions in consecutive mitoses (Noctor et al., 2004).

It has been proposed that for each cell division, parameters such as cell cycle kinetics, inputs from signalling pathways, interkinetic nuclear migration, transcriptional network status are integrated into the decision whether to execute self-renewing or neurogenic division (reviewed in Willardsen and Link, 2011). For example neural progenitors undergoing proliferative divisions have a longer S-phase compared to asymmetrically dividing progenitors (Calegari et al., 2005). Even though the, divisions of neural progenitors appear to be stochastic rather than predeterministic in nature, it is likely that the combination of both stochastic and deterministic inputs influences final decisions about the fate of the progeny

On the other hand, radial glia divisions follow a predetermined pattern in terms of neuronal subtype specification. Generation of the cortical projection neuron subtypes follows a precise temporal order (Shen et al., 2006) reviewed in Leone et al., 2008). Earlier in development neurons specific for the deep layers are generated, later in neurogenesis neurons of the upper layers are born. Therefore, over time, neurogenic potential of the radial glia becomes restricted, so that progenitors cannot produce neurons of the previous subtypes that were born earlier (Desai and McConnell, 2000; Frantz and McConnell, 1996; Shen et al., 2006).

However this long-standing view about temporal restriction of radial glia has been recently challenged. Elegant fate mapping experiments by Franco et al., 2012 have characterised subset of radial glia cells that express a marker of upper layer neurons Cux2. During early stages of neurogenesis Cux2-positive progenitors divide rather in a symmetric proliferative mode. At the time of upper layer production, they switch to asymmetric divisions to form upper layer neurons (Franco et al., 2012). These experiments indicate that subpopulations

of radial glia specified to produce neurons of different layers coexist already from the beginning of neurogenesis.

Another example is the temporal separation of neurogenesis and gliogenesis. In mammalian neocortex, neurogenesis always precedes gliogenesis (reviewed in Miller and Gauthier, 2007). Early neural progenitors strictly produce neurons and acquire the potential to give rise to the astrocytes later in development (Costa et al., 2009; Malatesta et al., 2000; Namihira et al., 2009).

To summarize, decisions to undergo symmetric or asymmetric divisions are flexible and do not follow a stereotyped pattern as in *Drosophila* larval brain, but temporal appearance of individual neuronal subtypes and astrocytes is predetermined.

Influence of individual factors on cell fate decision and how the asymmetry of the neurogenic divisions is generated will not be discussed in the introduction. Instead I will focus on Notch signalling, that is critical for cell fate decisions and the balance between proliferation and differentiation, which is directly relevant to the work presented in this thesis.

Notch Signalling

The core pathway

Notch pathway is a highly conserved signalling pathway, known to regulate cell fate decisions in various tissues reviewed in (Bray, 2006). The signalling occurs between the cells contacting each other, where one cell is expressing Notch receptors and other cell is expressing Notch ligands on the cell surface (Fig. 4). In mouse, there are four Notch receptors (Notch 1-4) and five ligands (Delta-like 1,3,4 and Jagged 1-2). Binding of the Notch ligand to the Notch receptor triggers a proteolytic cleavage of the receptor at two sites by ADAM metaloproteases and gamma-secretase complex. Activation of the cleavage is dependent on endocytosis of both the ligand and the receptor. Endocytosis of the ligand requires its ubiquitylation by the E3 ubiquitin ligase Mindbomb (Itoh et al., 2003; Koo et al., 2005). It has been proposed that pulling forces generated by endocytosis lead to

conformational changes in the receptor, exposing the cleavage sites for ADAM metaloproteases (Meloty-Kapella et al., 2012).

Cleavage of the Notch receptor by the gamma-secretase complex leads to the release of the Notch intracellular domain (NICD), which then translocates to the nucleus. In the nucleus, NICD associates with the CSL (CBF1/RBPJκ/Su(H)/Lag-1) and a transcriptional coactivator Mastermind-like to activate transcription of the target genes (Kopan and Ilagan, 2009).

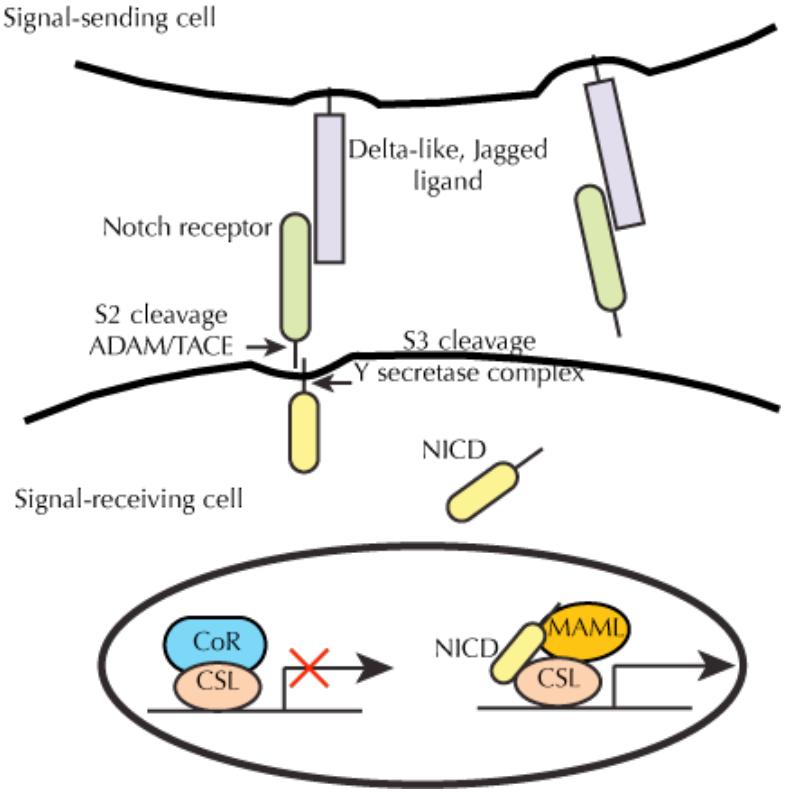


Figure 4. Notch signalling pathway. The pathway is activated by binding of the Notch receptor to the ligand expressed on the neighboring cell. Endocytosis of both ligand and the receptor allows cleavage of the Notch receptor at S2 site by ADAM metalloproteases. The truncated membrane-bound recetor fragment is cleaved by γ-secretase complex, releasing Notch intracellular domain (NICD). NICD translocates into the nucleus where it binds to the CSL DNA-binding protein. Association of NICD with CSL and Mastermind-like leads to derepression and activation of Notch target genes.

Notch signalling in the brain

Notch signalling is essential for the maintenance of the cortical progenitors. Conditional deletion of RBPj-k leads to premature neuronal differentiation and complete depletion of the progenitors (Imayoshi et al., 2010). Authors of the study further demonstrated that Notch signalling is required not only for the embryonic neural progenitors but also for the maintenance of the adult neural stem cells. Despite being crucial for the maintenance of the neural stem cells, Notch signalling is not needed for their initial formation, as it was shown in RBPj-k knockout mice (Hitoshi et al., 2002). Conversely, forced activation of the Notch pathway promotes progenitor fate at the expense of differentiated progeny (Hitoshi et al., 2002; Li et al., 2012b). Increase in the numbers of the progenitors is not accompanied by increased proliferation, suggesting that Notch promotes progenitor fate, but does not increase their proliferation (Chambers et al., 2001; Li et al., 2012b).

During neurogenesis, intermediate progenitors and newborn neurons have been described as a major source of Notch ligand (Yoon et al., 2008). This however opens a question, what is the source of Notch ligands and how is Notch signalling maintained in the neuroepithelial cells, before onset of the neurogenesis. Clearly, neuroepithelial cells require Notch ligand, since deletion of the Delta-like1 (Dll1), leads to loss of the Notch activity and premature differentiation (Kawaguchi et al., 2008).

Recently, an interesting model explaining the Notch signalling in early stages of cortical development has been proposed (Kageyama et al., 2009; Shimojo et al., 2011). In neural progenitors, key Notch target Hes1 is expressed in an oscillatory manner. The oscillatory expression is maintained by a negative feedback loop, where Hes1 binds and represses its own promoter (Takebayashi et al., 1994). Oscillations of Hes1 lead to oscillatory expression of the transcription factor Neurogenin2 and the Notch ligand Dll1, which are directly repressed by Hes1 (Shimojo et al., 2008). In addition, Neurogenin2 directly activates Dll1, ultimately leading to oscillatory expression of Dll1 in neural progenitors (Castro et al., 2006; Shimojo et al., 2008). This means that every progenitor shuffles between the state of being a Notch signal-sending and a Notch signal-receiving cell. Since oscillations of Hes1 in individual progenitors are not synchronized, the tissue is self-sufficient in maintaining Notch activity.

Radial glia also express Dll1 during the neurogenic phase of development, but the major source of the Notch ligand are intermediate progenitors and newborn neurons (Kawaguchi et al., 2008; Shimojo et al., 2008; Yoon et al., 2008). It has been shown that Mind bomb 1 (Mib1) ubiquitin ligase, required for ubiquitination and proper function of Dll1, is specifically expressed by intermediate progenitors and newborn neurons (Kawaguchi et al., 2008; Shimojo et al., 2008; Yoon et al., 2008).

Before the onset of neurogenesis, neuroepithelial/radial glial cells should also express Mib1, since these cells require Dll1 in order to maintain the Notch signalling and prevent premature differentiation (Kawaguchi et al., 2008). Shift in Mib1 expression from neuroepithelial cells/radial glia to intermediate progenitors and newborn neurons would result in displacement of the active form of Dll1 ligand from the radial glia to the differentiating progeny. Theoretically, this could be one of the factors contributing to the switch form symmetric to asymmetric divisions. After division of the radial glia, only one of the daughter cells inherits the basal process (Konno et al., 2008; Shitamukai et al., 2011). This cell shows high Notch activity and usually self renews (Shitamukai et al., 2011). The daughter cell that did not inherit the basal process would therefore lose contact with the source of Delta-like ligand in the SVZ, and commit to differentiate. Before the onset of neurogenesis, the cell losing the basal process could still receive the functional Delta ligand form the neighboring radial glia and therefore would re-establish the bipolar morphology. Indeed it has been shown that high activity of the Notch pathway promotes regrowth of the basal process of the radial glia (Shitamukai et al., 2011).

Transcriptional targets of Notch pathway

The best-characterised targets of the NICD/RBPj-k complex are Hes family of the transcriptional repressors (reviewed in Kageyama et al., 2008). Three members of the family Hes1, Hes3 and Hes5 are expressed in the neural progenitors where they act redundantly to promote progenitor fate. In mouse Hes1, Hes3, Hes5 triple-mutants all radial glia are lost due to premature differentiation (Hatakeyama et al., 2004).

Li et al., 2012 have performed genome-wide chromatin immunoprecipitation analysis coupled with RNA profiling of the cortices overexpressing Notch intracellular domain. Apart form the induction of the Hes proteins, the authors have described direct positive regulation of the transcription factors (Pax6, Sox2, Tlx) and signalling pathways that promote self-renewal such as Wnt and Shh pathway (Li et al., 2012b).

Important role of the Hes proteins in the neural progenitors is to repress the expression of the proneurogenic genes in the radial glia. In the dorsal cortex, Hes1 has been shown to repress a broad set of genes that promote neuronal differentiation and intermediate progenitor fate such as Neurod4, Neurogenin2, Dll1, Hes6, Eomes (Tbr2), Insm1 (Shimojo et al., 2008).

Although Hes genes are critical for maintenance of neural progenitors, conditional deletion of all three Hes1, 3, 5 genes in the dorsal cortical progenitors using Emx1-cre shows only mild defects in cortical layering (Imayoshi et al., 2008). The authors proposed that in neocortex, loss of Hes 1, 3, 5 genes is compensated by the upregulation of Hey 1 and Hey 2 repressors.

AIM AND DESIGN OF THE STUDY

Control of proliferation and differentiation of the neural progenitors during embryonic development determines the number of neurons in the adult brain. But, how the decisions whether to proliferate or to differentiate are made is not well understood. The goal of this study is to discover novel factors regulating the maintenance of the neural progenitors in the murine embryonic neocortex.

As a first step we established a FACS purification protocol that allows rapid isolation of progenitor and neuronal cells from embryonic neocortex. The sorted cells were used to examine gene expression profiles of progenitors and neurons by high-throughput RNA sequencing. Next, we conducted analysis of the transcriptome profiles combined with candidate approach screening to discover new genes involved in the maintenance of the

neural progenitors. The last part of the Results will describe the two novel genes essential for proper cortical development.

RESULTS

FACS purification of progenitors and neurons from embryonic neocortex

FACS purification strategy

Several approaches can be utilized to isolate neural progenitors or neurons from the embryonic brain. Dissociated neural tissue can be cultured in selective conditions, which promote growth of neural stem cells or neurons (Pollard et al., 2006). However, growing cells *in vitro* puts them out of their natural environment and they do not fully recapitulate characteristics of the radial glia in the dorsal cortex. For example, exclusive neurogenic potential and regional identity tend to be lost in *in vitro* cultures (reviewed in Conti and Cattaneo, 2010).

In order to be able to work with authentic cortical progenitors and neurons, we set to establish a FACS-based methodology that would allow purification of progenitors and neurons directly from the developing neocortex. In the hematopoietic system, individual cell types can be precisely labeled by a set of surface markers. But in the neural lineage, surface markers available for the identification of different cell types are very limited.

To label the radial glia, we took advantage of the Dty54 transgenic mice (Tyas et al., 2006) that express GFP under the control of human Pax6 promoter (Fig. 5A). Human Pax6 promoter fully recapitulates the expression of mouse Pax6 promoter (Tyas et al., 2006). Pax6 is a homeodomain transcription factor specifically expressed in radial glia of the dorsal but not ventral telencephalon. Analysis of the GFP expression in E14.5 cortex of DTy54 mice by flow cytometry shows two separate cell populations consisting of GFP negative and GFP positive cells (Fig. 5B). To assess suitability of the GFP expressed from human Pax6 promoter for labeling of cortical radial glia, we examined the overlap between the expression of Pax6 and GFP in E14.5 brains. The strongest expression of GFP is visible in Pax6 positive radial glia of the VZ (Fig. 5C). However GFP is also detectable,

albeit at lower levels in the SVZ and IZ. This indicates perdurance of the GFP from radial glia into the intermediate progenitors and newborn neurons. Neurons in the cortical plate are GFP negative. The GFP signal visible in the cortical plate originates from basal processes of the radial glia.

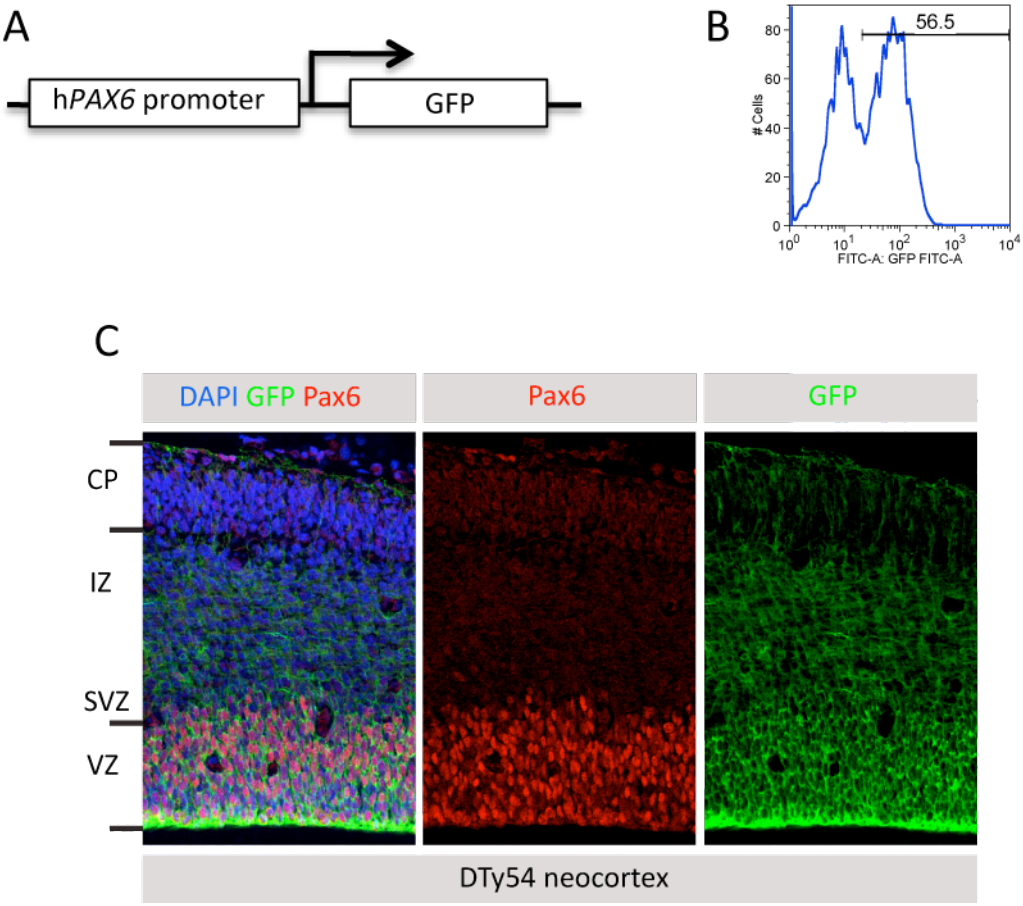


Figure 5. DTy54 mice express GFP under control of Pax6 promoter. A. Scheme showing construct inserted in DTy54 mice. GFP is expressed from human Pax6 promoter. **B.** Flow cytometry analysis of the DTy54 transgenic E14.5 neocortex. Two cell populations, GFP+ and GFP- can be distinguished based on the GFP fluorescence. **C.** Coronal section of E14.5 neocortex stained for Pax6 and GFP. The domain of strongest GFP expression largely overlaps with Pax6 staining in the ventricular zone. Cells in the intermediate zone show lower levels of GFP. Neurons in the cortical plate are GFP-. Note processes of the radial glia in the cortical plate.

Initial attempts to purify radial glia were based on the intensity of the GFP signal, assuming that cells having high levels of GFP would be radial glia and GFP negative cells would be representing neurons. We dissected cortices from E14.5 embryos and separated them into

GFP negative and GFP positive cells by FACS (Fig. 6A). To determine the identity of the FACS-purified GFP positive and GFP negative cell populations, the cells were grown for two hours on laminin/fibronectin coated coverslips and stained for radial glial and neuronal markers. The GFP positive cell population contained many GFP+ Tuj1 positive neurons, showing neuronal contamination of putative radial glial population (Fig6B). Gating on cells expressing highest levels of GFP did not significantly improve purity of the sorted GFP+ cells. This result means that due to the stability of the GFP, newborn neurons that inherited GFP from radial glia are co-purified together with the progenitors.

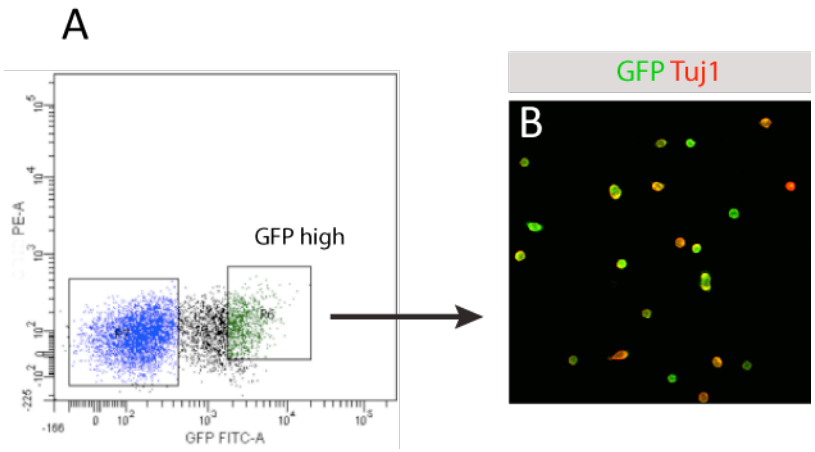


Figure 6. GFP signal in DTy54 neocortex is not sufficient to isolate neural progenitors.
A. E14.5 DTy54 neocortices were dissociated into single cell suspension and analysed by FACS. Representative dot plot showing gating for GFP highly positive cells. GFP negative cells are shown in blue. **B.** The sorted cells were stained for neuronal marker Tuj1. Fraction of the GFP+ cells is positive for Tuj1, showing copurification of neurons.

To achieve better discrimination between progenitors and neurons in Dty54 cortices, we devised an alternative strategy using two additional surface markers CD133 and L1Cam. In developing neocortex, CD133 is enriched at ventricular surface marking apical endfeet of radial glia (Fig. 7A). The second marker, L1cam is expressed in neurons, playing important role in migration and axon guidance. In E14.5 cortex, neurons in the intermediate zone and cortical plate express L1cam. Axonal tracts in the intermediate zone are also stained by L1cam antibody. Pax6 positive radial glia in the ventricular zone are however L1cam negative (Fig. 7B,C).

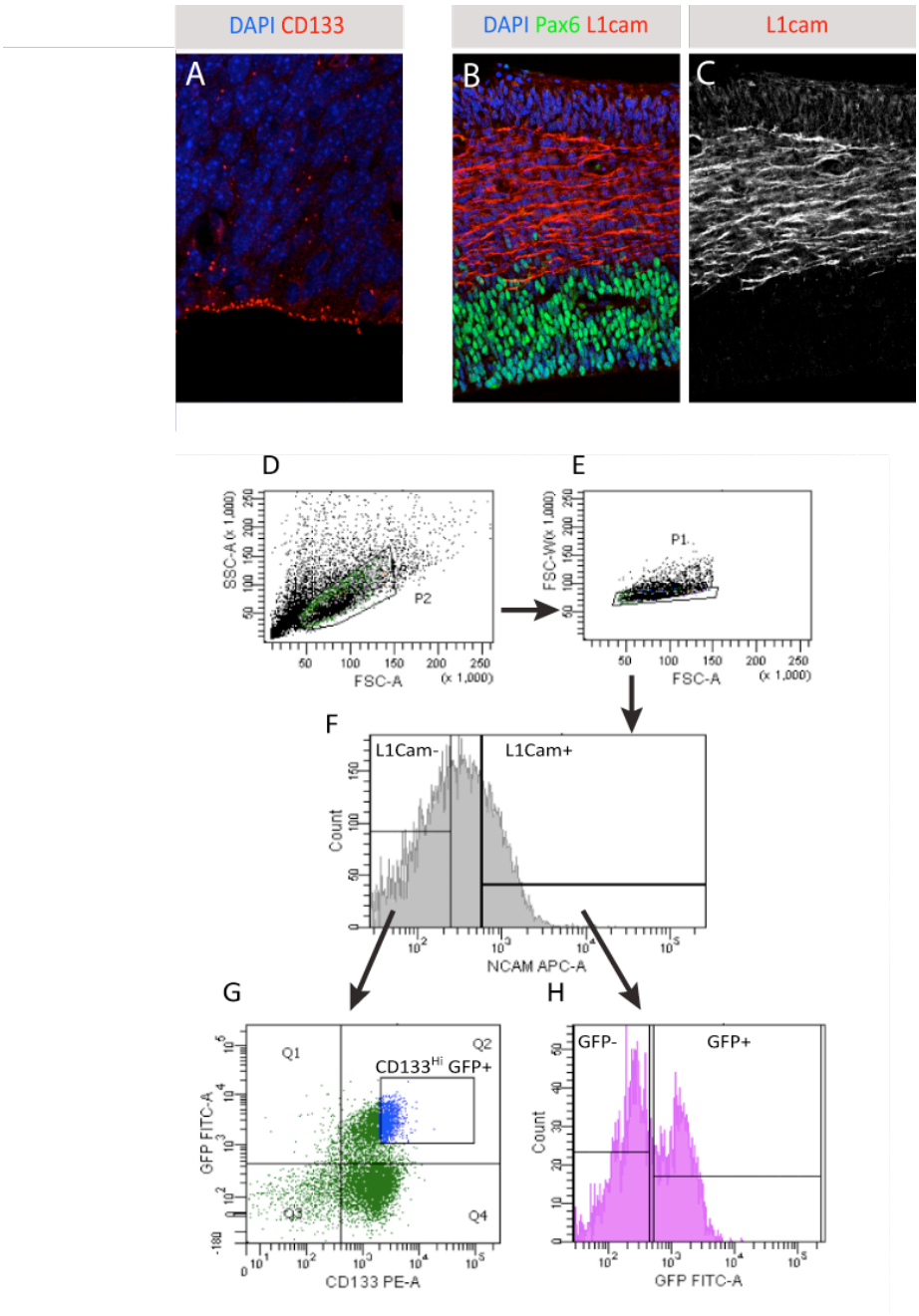


Figure 7. FACS strategy for isolation of progenitors and neurons. A. E14.5 ventricular zone of the dorsal pallium. C133 staining is enriched at the ventricular surface in the apical endfeet of the radial glia. **B-C.** Pax6 and L1cam immunolabelling of E14.5 neocortex. L1cam staining marks neurons in the intermediate zone and cortical plate. Note L1cam positive axons in the intermediate zone. L1cam does not stain Pax6 positive radial glia in the ventricular zone. **D-H.** Gating strategy using CD133 and L1cam labelling of DTy54 cortices. **D.** Cell were separated from debris using forward and side scatter. **E.** Cell doublets were excluded according to width of the forward scatter peak. **F.** separation of cells into L1cam positive and negative population. **G.** CD133^{hi} GFP+ progenitors in L1cam negative population. **H.** GFP- and GFP+ positive neurons of L1cam population.

The setting of the FACS gates was performed in the following way. Forward and side scatter was used to separate cells from the cell debris (Fig. 7D). Single cells and cell clumps consisting of two or more cells were distinguished based on the width of the forward scatter peak (Fig. 7E). Single cells were separated into L1cam negative and L1cam positive population (Fig. 7F). L1cam+ gate in composed of GFP+ and GFP- cells presumably containing GFP+ neurons of the intermediate zone and GFP- neurons of the cortical plate, respectively (Fig. 7H). L1cam negative cells were plotted as GFP vs. CD133 dot plot (Fig. 7G). Putative neural progenitors were gated as GFP+/CD133^{hi} population.

Characterisation of the FACS-sorted progenitors and neurons

In order to determine the composition of the sorted populations, cells were grown in culture for 2 hours to allow attachment to the coverslips and analysed by immunostaining for progenitor and neuronal markers. Among the GFP+/CD133^{hi} population, 99% of the cells were positive for GFP, whereas GFP-/L1cam+ cells contained contamination of 1.1% of the GFP+ cells (Fig. 8A-E). Vast majority of the GFP+/CD133^{hi} cells were positive for markers of radial glia Nestin, Pax6 and Sox2 (Fig. 8B-D). Within the GFP-/L1cam+ gate, minor fraction of the cells expressed Pax6 Nestin or Sox2 (Fig. 8F-H).

To label neurons, the sorted cells were stained for neuronal markers Map2 and Tuj1. Only few of the GFP+/CD133^{hi} containing cells were stained positive for the neuronal markers Map2 and Tuj1 (Fig. 8K,L), compared to prominent Map2 and Tuj1 expression in the GFP-/L1cam+ population (Fig. 8O,P). Antibody against proliferation marker Ki67 strongly stained GFP+/CD133^{hi} cells but not cells of the GFP-/L1cam+ population (Fig. 8I-M).

Taken together, GFP+/CD133^{hi} cells predominantly contain radial glial cells, as evidenced by positive staining for Pax6, Nestin, Sox2 and Ki67 and largely absent expression of neuronal markers. Within the GFP-/L1cam+ population, majority of the cells are postmitotic neurons expressing Tuj1 and Map2. These cells do not stain for progenitor-specific or proliferation markers. To determine whether sorted radial glial cells also contain intermediate progenitors, GFP+/CD133^{hi} cells were stained for intermediate progenitor marker Tbr2 (Fig. 8J,N). About 37% of the GFP+/CD133^{hi} cells expressed Tbr2. Since majority of the GFP+/CD133^{hi} cells (>97%) also express RG makers, this indicates that Tbr2 positive cells are radial glia commited to become an intermediate progenitor. This

resembles *in vivo* situation where 30% of the radial glia are estimated to be in transition towards the intermediate progenitor fate (Arai et al., 2011).

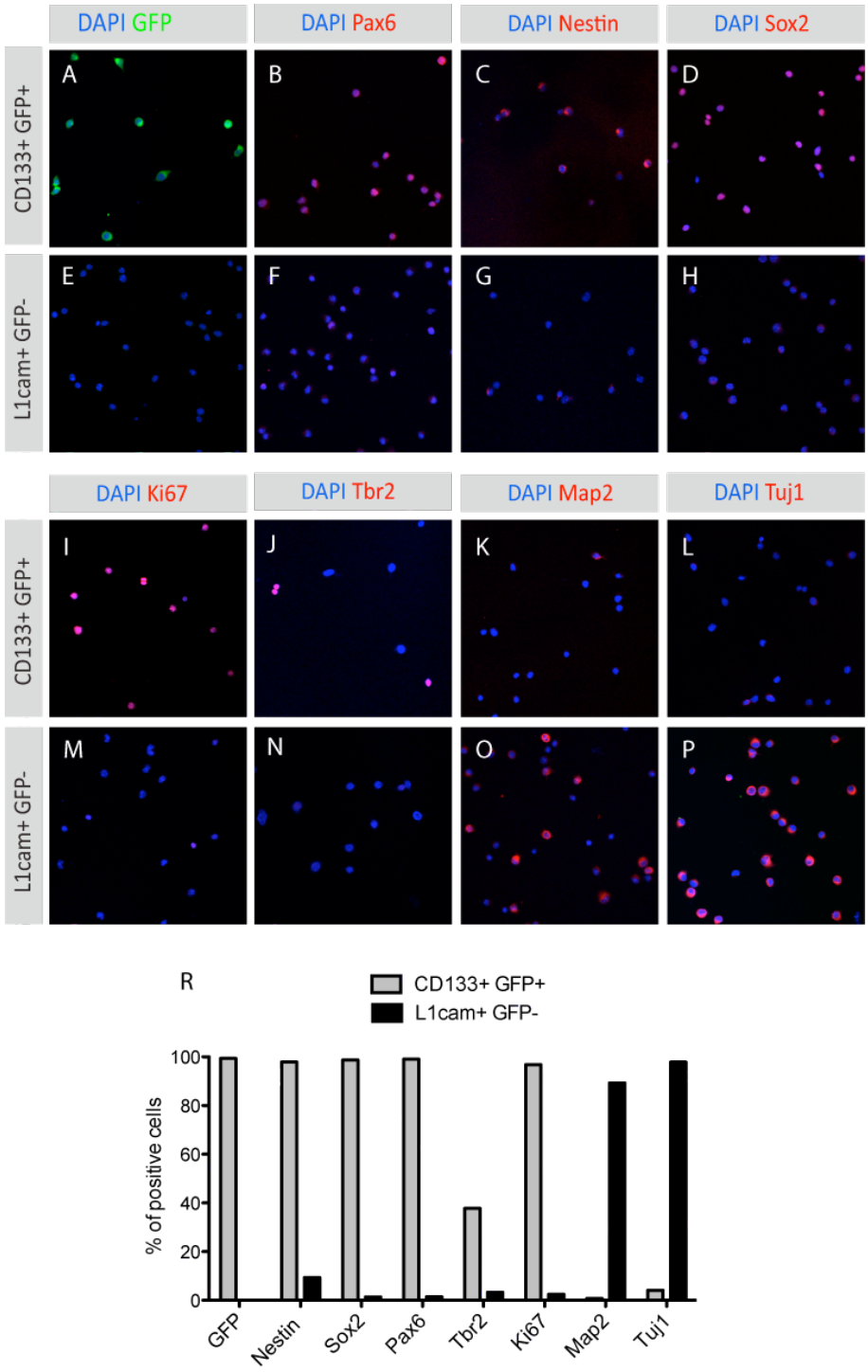


Figure 8. Immunofluorescence staining of sorted for progenitor and neuronal markers. CD133+ GFP+ cells **(A)** are positive for progenitor markers Pax6, Nestin, Sox2 and proliferation marker Ki67 **(B, C, D, I)**, but negative for neuronal markers Map2 and Tuj1 **(K, L)**. L1cam+ GFP- cells **(E)** are negative for progenitor markers and Ki67 **(E, F, G, H, M)** and positive for neuronal markers Map2 and Tuj1 **(O, P)**. Fraction of CD133+ GFP+ cells express intermediate progenitor marker Tbr2 **(J)**, whereas L1cam+/GFP- cells do not express Tbr2 **(N)**. Quantification of marker stainings **(R)**.

To extend characterization of the sorted cells and assess their suitability for transcriptional profiling, we analysed the expression pattern of the progenitor and neuronal markers on mRNA level by quantitative PCR. Progenitor specific genes Nestin, Pax6, Glast, Eomes were highly upregulated in CD133^{hi}/GFP+ cell population (Fig. 9). Higher expression of neuronal markers Tubb3 and L1CAM was detected in L1cam+/GFP+ cells, compared to CD133^{hi}/GFP+ cells.

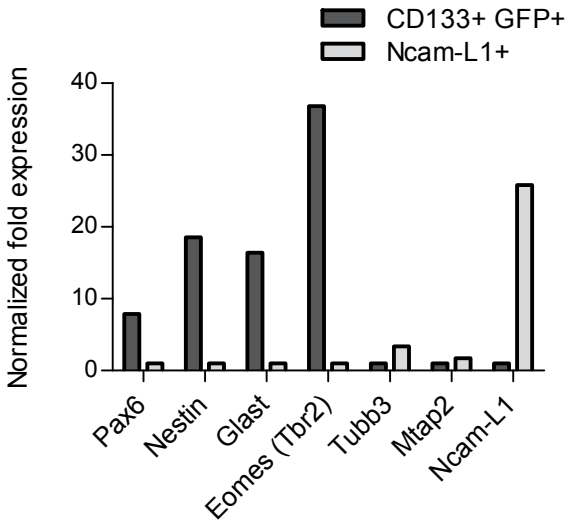
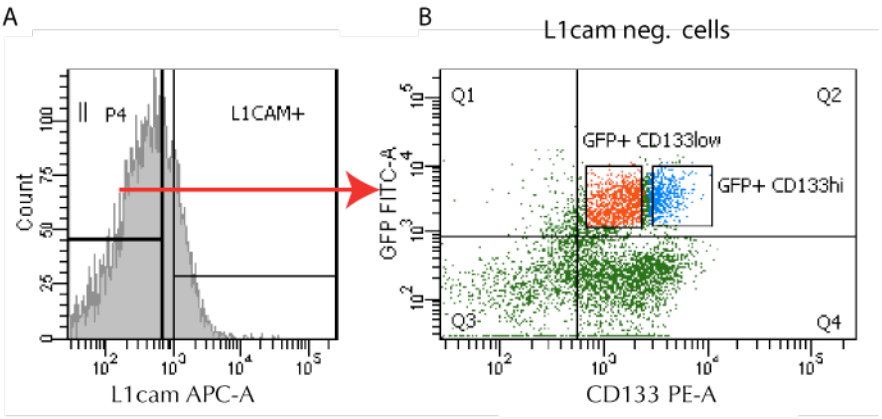


Figure 9. Expression of progenitor and neuronal markers in FACS-sorted cells. CD133+/GFP+ cells express higher levels of progenitor-specific genes Pax6, Nestin, Glast and Tbr2 than L1cam+/GFP- cells. Expression of neuronal markers Tubb3, Mtap2 and L1cam is upregulated in L1cam+/GFP+ cells.

Taken together, the above-described FACS purification strategy allows separation of two cell populations from the embryonic neocortex that are highly enriched for radial glia and neurons.

Gating according to the intensity of CD133 expression limits yields of progenitors purified per sort. To examine the differences between the GFP+ progenitors expressing different levels of CD133, we separated CD133hi/GFP+ and CD133low/GFP+ progenitors by FACS (Fig. 10A,B). Expression of progenitor and neuronal markers in these two cell populations was compared by qPCR. Markers of radial glia Pax6, Nestin and Glast were expressed at higher levels in CD133hi/GFP+ cells (Fig. 10C). In contrast, CD133low/GFP+ cells expressed higher levels of IPC marker Eomes (Tbr2) and neuronal markers L1cam and Tubb3 meaning that GFP positive population expressing low levels of CD133 contains more cells committed to differentiate. This result shows that sorting according to the levels of CD133 is an important factor that allows enrichment of the radial glia and separation of more differentiated progeny.



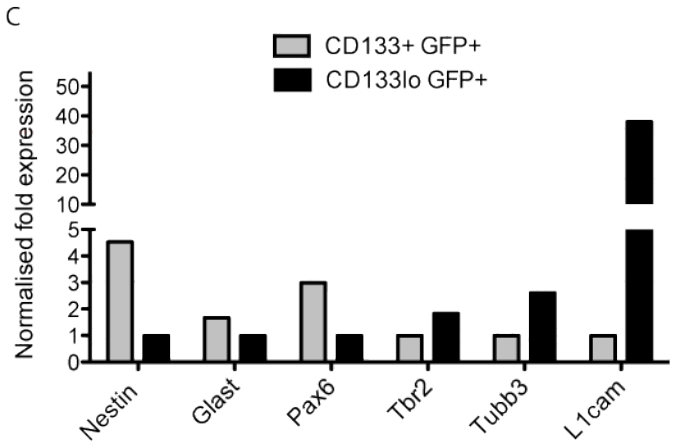


Figure 10. Characterisation of progenitors expressing various levels of CD133. A,B. L1cam negative cells were separated into CD133 low/GFP+ cells and CD133hi/GFP+ cells. **C.** Radial glial-specific genes Nestin Glast, Pax6 are highly expressed in CD133hi/GFP+ progenitor cells. CD133low/GFP+ cell show upregulated expression of intermediate progenitor marker Tbr2 and neuronal markers Tubb3 and L1cam. CD133low/GFP+ cell population is enriched for differentiating cells comparing to CD133hi/GFP+ cells.

Self-renewal and differentiation of sorted cells *in vitro*

A characteristic feature of the radial glia is the ability to self-renew and produce neurons. To address whether FACS purified progenitors comply with this criteria the cells were grown in medium supporting self-renewal or in conditions promoting neuronal differentiation. When grown for 5 days in neural stem cell medium NSA (supplemented with bFGF, EGF, N2) the sorted progenitors expanded and formed a population of cells positive for the radial glial markers Sox2 and Nestin, but negative for neuronal Tuj1, demonstrating self-renewal capacity of the sorted progenitors (Fig11. A,B). When grown in differentiation medium (NBM suppl. with N2, B27, bFGF) Tuj1 positive neurons were detected, confirming the ability of the FACS-sorted progenitors to differentiate into neurons (Fig. 11C). On the other hand, colonies of neural stem cells were not detected in cultures of FACS-purified neurons suggesting that few progenitors present in the culture were unable to propagate.

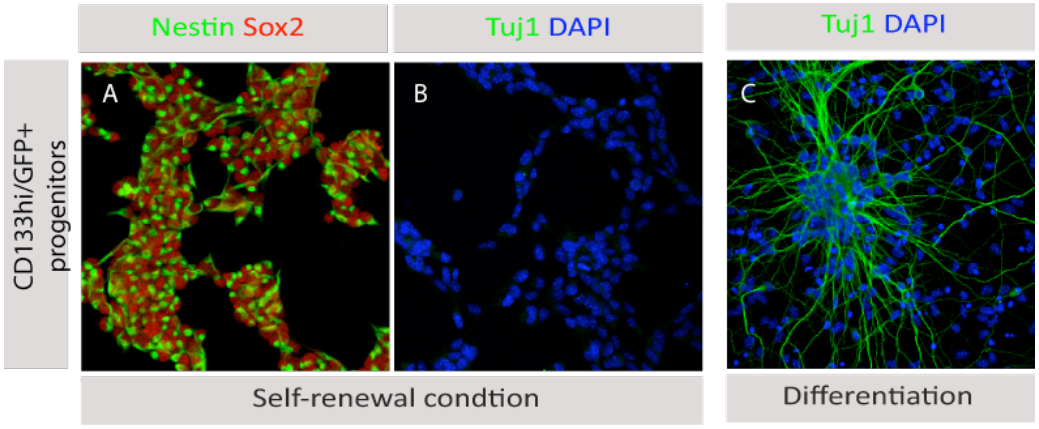


Figure 11. Self-renewal and differentiation of FACS-sorted progenitors *in vitro*. CD133hi/GFP+ cells grown in stem cell medium for 5 days form pure population of neural stem cells positive for RG markers Nestin, Sox2 **(A)** and negative for neuronal Tuj1 **(B)**. Cultivation of cells under differentiating conditions promotes differentiation into Tuj1 positive neurons **(C)**.

Transcriptome profiling of cortical progenitors and neurons

To identify the genes expressed in the cortical neural progenitors and neurons, we performed transcriptome profiling by next-generation RNA sequencing (RNA seq) using Solexa technology (Illumina). Transcriptome profiling by RNA seq extends beyond the capabilites of microarrays, allowing simultaneous analysis of gene expression and splicing, while providing nucleotide level resolution.

The aim of performing the transcriptome profiling of cortical progenitors and neurons was to use the data as a resource for the identification of new regulators of neurogenesis in the developing mouse brain.

RNA seq of E14.5 cortical neural progenitors and neurons

To collect sufficient amounts of starting material for cDNA library preparation 3-5 sorts of E14.5 litters were pooled together. Isolation of total RNA from progenitors and neurons was followed by purification of polyA+ RNA. PolyA+ RNA was fragmented to size between 100-500 nt and used as a template for first and second strand cDNA synthesis. The cDNA libraries from two biological replicates of progenitors and neurons were sequenced by Solexa technology (Illumina) generating 76bp paired-end reads. We obtained in between 14 and 35 milion reads per sample (Fig. 12A). Around 2.5-5 million reads per sample could not be mapped to any sequence in the mouse genome and were likely a product of the sequencing errors. Only small fraction of the reads was mapped to several genomic locations and therefore excluded from the analysis. Gene expression (FPKM value) was quantified by calculating number of sequenced fragments per kilobase of the gene (excluding introns) per million mapped reads.

Correlation coefficients of the FPKM values for biological replicates were 0.86 and 0.98 for progenitors and neurons respectively (Fig. 12B). Somewhat lower correlation between biological replicates of progenitor samples could be explained by the variability in the ratio of self-renewing and differentiating progenitors. Intensity of CD133 staining was used to distinguish between these two cell types. But since levels of CD133 staining were different for each sorting, CD133hi/GFP+ gate for progenitors had to be adjusted individually for

each sort. This could have possibly resulted in different ratios of self-renewing and differentiating progenitors that have already upregulated transcription of differentiation-associated genes. However, this did not have a negative effect on downstream processing of the RNA seq data.

To learn about differences in gene expression between progenitors and neurons, we performed a differential gene expression analysis. We identified 3865 differentially expressed genes (log2 fold change>1, adjusted p value<0.01) and FPKM value at least 1 in either progenitor or neuronal dataset. 1993 genes were expressed significantly higher in progenitors and 1872 higher in neurons. These numbers of differentially expressed genes are similar to recently published transcriptome sequencing of laser-capture microdissected progenitor and neuronal regions of the mouse embryonic neocortex (Ayoub et al., 2011; Fietz et al., 2012).

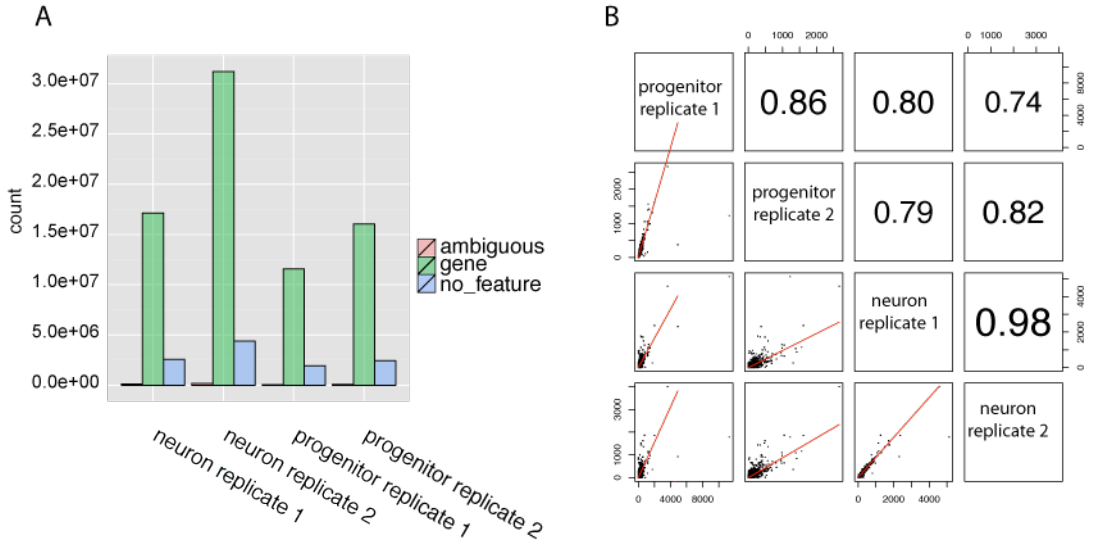


Figure 12. Quality of the sequnecing reads A. Numbers of reads which can be mapped to the genome (green). Ambiguous reads are mapped to more that one location (pink). Reads that cannot be mapped to the genome are shown in blue. **B.** Correlation of FPKM values between the samples and replicates.

Validation of the transcriptome data

To validate quality of the dataset, we analysed whether expression of marker genes follows the expected pattern. Radial glia markers Glast, Nestin, Prominin 1, Pax6 and Sox2 were

expressed at higher levels in progenitor dataset (Fig. 13A). Higher expression of neuron-specific genes such as Dcx, L1cam, Synapsin 1, Tbr1 and Tubb3 was detected in neuronal samples. Transcription factor Tbr2 (Eomes gene) was upregulated in progenitor sample (log2FC=2.56). This is to be expected, since the sorted radial glia contain cells already committed to intermediate progenitor fate.

To assess whether we can detect biological changes characteristic for neuronal differentiation, we examined the activity of Notch pathway in our RNA seq data. Notch activity is high in radial glia, where it is essential to prevent premature differentiation, but low in intermediate progenitors and neurons (Mizutani et al., 2007). To ask whether this is correctly reflected in our transcriptome data, we analyzed the expression of several pathway members in progenitor and neuronal samples (Fig. 13A). Notch receptors were expressed at higher levels in progenitors than neurons. Similarly two downstream targets Hes1 (log2FC=5.36) and Hes5 (log2FC=4.88) were also upregulated showing higher activity of Notch signalling in the progenitor dataset.

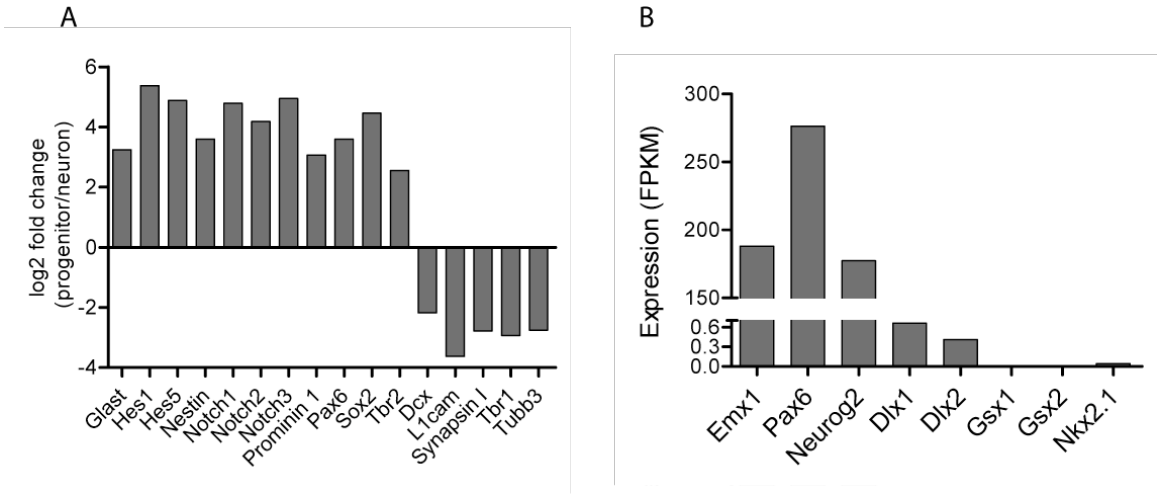


Figure 13. Verification of marker-specific expression in RNA seq data. A. Progenitor-specific markers Glast, Nestin, Promini-1, Pax6, Sox2 and Tbr2 are upregulated in progenitors. Neurons show higher expression of neuron-characteristic genes Dcx, L1cam, Synapsin I, Tbr1, and Tubb3. High Notch activity in progenitors in evident from upregulated expression of Notch1-3 receptors and Notch target genes Hes1 and Hes5. (log2 fold change >1, FDR 1%) **B.** Dorsal pallial markers are highly expressed in sequenced progenitors, whereas genes specific for progenitors of the ventral telencephalon are not detected or expressed at very low level (FPKM<1).

To examine regional identity of the sequenced progenitors, we looked at expression levels of the dorso-ventral patterning genes. Markers of the neocortex Emx1, Pax6 and Neurog2 were highly expressed (FPKM<177) in progenitors, whereas transcripts specific for progenitors of the ventral telencephalon such as Dlx1, Dlx2, Gsx1, Gsx2 and Nkx2.1 were either not expressed or detected at very low levels (FPKM<1) (Fig. 13B). This confirms correct pallial identity of the progenitor sample.

Additionally, set of differentially expressed genes was checked against publicly available in situ hybridization databases (ISH) of the developing brain (Genepaint, Allen Brain Atlas). In general, the expression patterns correlated well between the ISH databases and transcriptome data. Cross-referenced genes that were expressed at higher levels either in progenitors or neurons did not show opposite expression pattern in the in situ brain atlases.

To summarize, characteristic markers of radial glia and neurons and are all expressed in correct pattern, confirming the quality of the transcriptional profiles of FACS-sorted progenitors and neurons obtained by RNA sequencing. Thus, the progenitor and neuronal transcriptomes can be reliably used for more comprehensive data mining.

GO term analysis

To reveal which biological processes are enriched in progenitors and neurons, we performed a GO term analysis of subsets of genes that are upregulated in progenitors or neurons using DAVID Bioinformatics Resources (Huang da et al., 2009a, b). GO term categories such cell cycle, cell division, DNA replication or DNA repair were overrepresented in progenitors (Table 1). This is expected, since neural progenitors are actively proliferating cells.

In neurons, GO terms such as neuron projection development, neuron differentiation, axonogenesis or cation transport were significantly enriched. These neuron-specific processes confirm neuronal phenotype of the sequenced cells.

Table 1. GO term analysis of biological processes enriched in progenitors and neurons. GO terms characteristic for proliferating cells are overrepresented in progenitors. Processes such as neuron differentiation, axonogenesis or synaptic transport are enriched in neurons.

Progenitors		Neurons	
GO term (Biological Process)	p value	GO term (Biological Process)	p value
cell cycle	9.1E-68	neuron projection development	6.1E-25
cell cycle process	1.9E-49	cell projection organization	3.0E-20
cell cycle phase	1.7E-48	synaptic transmission	4.6E-20
cell division	1.5E-45	neuron development	3.7E-20
mitotic cell cycle	2.8E-44	neuron differentiation	8.8E-20
M phase	1.1E-42	transmission of nerve impulse	1.8E-18
DNA metabolic process	7.0E-39	neuron projection morphogenesis	1.8E-18
M phase of mitotic cell cycle	5.9E-38	axonogenesis	4.1E-18
nuclear division	5.1E-37	metal ion transport	1.2E-19
mitosis	5.1E-37	ion transport	4.0E-19
organelle fission	2.0E-36	cell part morphogenesis	5.5E-18
DNA replication	1.7E-26	cell-cell signalling	1.1E-17
response to DNA damage stimulus	3.9E-25	cation transport	1.1E-17
DNA repair	6.9E-25	potassium transport	1.9E-14
chromosome segregation	1.9E-22	cell morphogenesis	2.3E-14

Identification of candidate self-renewal genes

Integration of transcriptome profiling with functional data

Transcriptional profiling provides genome-wide information about gene expression levels. One issue that arises, when handling such large datasets, is how to extract the required information. The question being asked in this case is how can transcriptional profiling be used to identify novel factors required for self-renewal of neural progenitors in the developing murine neocortex.

Recently, our lab has performed a genome-wide RNAi screen of self-renewal in *Drosophila* larval neural stem cells, the neuroblasts (Neumuller et al., 2011). The screen has identified hundreds of genes that upon knockdown cause defects in *Drosophila* neural lineage. Majority of the genes showing RNAi phenotype in larval neuroblasts have a mammalian orthologue and represent a valuable source of new candidate genes regulating self-renewal in developing mouse central nervous system.

Our strategy was to take advantage of the functional data from the RNAi screen and integrate it with gene expression profiling of murine neural progenitors and neurons.

As a first step, we identified mouse orthologues of all genes that gave a phenotype in the screen. We could find 538 mouse homologues of the genes from the Drosophila RNAi screen. If the mouse homologue has a potential function in the developing brain it should be expressed in the tissue in first place. Strikingly, out of 538 genes, 492 were expressed at FPKM>1, which is generally used as a cut-off value and covers virtually all known genes that are involved in the regulation of neurogenesis.

To narrow down the list, we made an assumption that many of the putative self-renewal factors should be expressed at higher levels in progenitors than in neurons. Therefore we looked how many of the mouse homologues are upregulated in progenitors at log2FC>1 (FDR 1%). We identified 84 genes that follow the expected expression pattern. The list contained 8 genes already known to be required for proper brain development such as transcription factors Ascl1, Prox1 and Sall1, actin nucleator Diap2, histone methyltransferase Suz12 and Numb (Harrison et al., 2012; Miro et al., 2009; Rasin et al., 2007; Thumkeo et al., 2011). This means that almost 10% of the genes from the list are represented by already known regulators of neural development. This remarkably high value demonstrates conservation of the key mechanisms that control neurogenesis between the two species.

To analyze which biological processes are overrepresented in this group we performed the GO term analysis using DAVID Bioinformatics Resources (Huang da et al., 2009a, b). The set of genes was most significantly enriched for terms DNA replication, cell division, cell cycle and mitosis (p-value< 5.5E-16). This indicates that the overlap between the genes that are upregulated in mouse progenitors and gave a phenotype in the *Drosophila* screen, is mainly represented by the genes generally required for proliferation. To find new candidate regulators of self-renewal, the list was manually annotated in the following way. All genes that have a known general role in cell cycle or mitosis were excluded. Next, all genes that were already published to have a role in the developing CNS and regulators of the signalling pathways that have a known function in the nervous tissue were removed

together with the genes required for cell survival. However, using these criteria no candidate genes were chosen for further characterization.

Selection of candidate genes for RNAi screening

To assemble the list of candidate regulators of self-renewal in neural progenitors, we used the following strategy. Subset of genes that are expressed higher in progenitors than neurons (log2 fold change>2, FPKM>1, FDR<1%), were used as an input for literature search. Genes involved in the processes such as cell cycle progression, DNA replication and cell division, together with genes already described to have a function in the developing brain were excluded from the list. In short, aim of the search was to find differentially expressed uncharacterised genes and genes involved in the processes that were not reported before to be important for brain development. The first eight genes from the list were tested by RNAi knockdown in radial glia of the E14.5 neocortex (Table 2).

Table 2. Set potential regulators of neurogenesis tested by RNAi knockdown in radial glia.
log2FC (log2 fold change), adj. p value (p value adjusted according to Benjamini), FPKM normalized expression value (fragments per kilobase per million mapped reads)

Gene symbol	Log2 FC	adj. p value	FPKM
Ddah1	3.65	1.12E-58	80.6
Depdc1b	5	8.20E-55	17.51
Pcgf5	4.69	1.77E-26	7.42
Ptgfrn	4.68	2.12E-104	101.86
Tis11b	5.08	3.75E-105	190.48
Tis11d	2.76	1.44E-25	37.38
Tox3	3.62	9.59E-64	75.83
Zfp367	4.89	1.29E-45	33.14

Candidate approach screening *in vivo*

To investigate the role of the selected candidate genes in murine cortical development, we performed RNAi-mediated knockdown *in vivo*. Anatomical organization of the developing central nervous system offers remarkable possibilites for gene delivery into the neural progenitors *in vivo*. Lateral ventricles of the embryonic forebrain can be injected with viruses or plasmids. Layered organization of the embryonic neocortex, where radial glia

progenitors are in contact with the ventricles and differentiated progeny is away from the ventricular surface, allows delivery of the transgene specifically into the progenitor cells.

In utero electroporation

The technique of plasmid delivery into the radial glia progenitors is called in utero electroporation and has been successfully used in mouse or rat embryos (Shimogori and Ogawa, 2008). In this surgical procedure, uterine horns are exposed via small midline incision on the abdomen of the pregnant dam under general anesthesia (Fig. 14A). Thin pulled glass capillary is used to inject plasmid DNA through the uterine wall and amniotic sack into the lateral ventricle of the forebrain. Usually, non-toxic tracing dye such as Fast Green is used to track the injection progress. To introduce the plasmid DNA into the radial glia, electrode pads of the tweezer-type electrode are positioned in such a way that the anode is placed on the dorso-lateral area of the injected brain hemisphere. Series of electric pulses transfer the DNA into the neural progenitors. In order to trace the electroporated cells, a plasmid encoding a fluorescent protein is added to each plasmid DNA. After electroporating the desired number of embryos, the uterine horns are placed back into the abdominal cavity. The abdomen is filled with warm saline and sutured with surgical silk or dermal stapler. After the surgery, the mouse is kept under surveillance and embryos are allowed to develop normally for the required period of time.

Example of cortex electroporated at E14.5 and fixed three days later is shown in (Fig. 14B). Electroporated cells are distributed throughout the whole cortical thickness. Radial glia and intermediate progenitors are located in ventricular and subventricular zone (VZ/SVZ), newborn migrating neurons are in the intermediate zone (IZ), and neurons reaching final destination are located in the cortical plate (CP). Tangentially oriented GFP positive fibers are callosal projections of the electroporated neurons (Fig. 14B). This distribution pattern of electroporated cells is highly reproducible between the experiments allowing reliable assessment of the phenotypes. Position and relative distribution of the electroporated cells provides clue about the possible phenotype. For example, reduction in numbers of cells from the VZ/SVZ and accumulation of the cells in the IZ+CP indicates premature differentiation. Accumulation of the cells in the VZ/SVZ can point towards increase in symmetric proliferative divisions. Neuronal migration defects can be manifested by altered

distribution of the neurons between the IZ/CP. Therefore, no additional stainings of the sectioned electroporated brains are required for preliminary assessment of the phenotype.

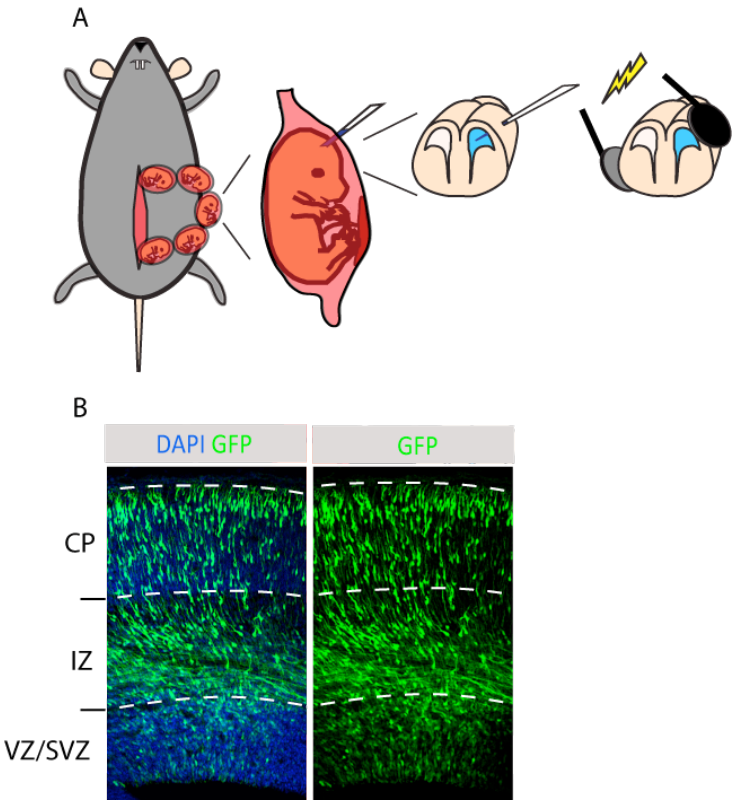


Figure 14. In utero electroporation. A. Scheme of the procedure. Lateral ventricle of the mouse embryo is injected with plasmid DNA. Electric pulse applied with tweezer electrodes transfers DNA into the radial glia lining the ventricle. **B.** E17.5 neocortex electroporated with GFP expression vector at E14.5. Electroporated progenitors are in the VZ/SVZ. Differentiated neurons originating from the VZ/SVZ progenitors are locared in the IZ and CP. ventricular/subventricular zone (VZ/SVZ), intermediate zone (IZ), cortical plate (CP).

Knockdown of candidate genes *in vivo*

To assess the role of candidate genes selected for in vivo testing (Table. 2), we cloned the V5-tagged open reading frames (ORF) of the genes and used them for testing the knockdown efficiency of the RNAi constructs in HEK 293T cells. We performed in utero electroporation of RNAi and GFP plasmid at E14.5 and analysed the phenotype three days later at E17.5.

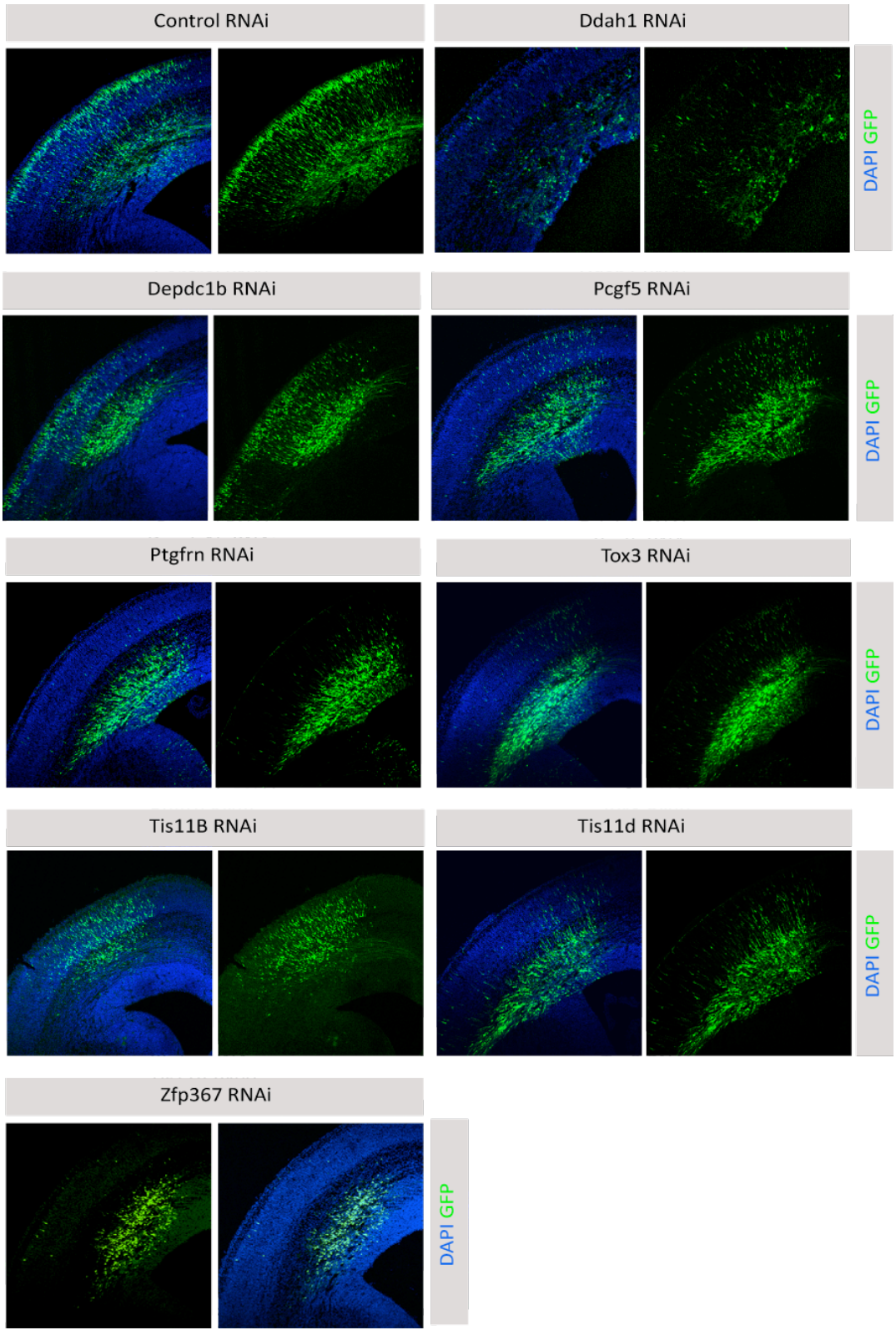

Control RNAi
Ddah1 RNAi
DAPI GFP
Depdc1b RNAi
Pcgf5 RNAi
DAPI GFP
Ptgfrn RNAi
Tox3 RNAi
DAPI GFP
Tis11B RNAi
Tis11d RNAi
DAPI GFP
Zfp367 RNAi
DAPI GFP

Figure 15. Knockdown of candidate genes in radial glia of the neocortex. RNAi constructs with GFP expression vector were electroporated at E14.5. Distribution of GFP+ cells was analysed in coronal sections of E17.5 brains counterstained with DAPI to label nuclei. Brains electroporated with Pcgf5, Ptgfrn, Tox3, Tis11d and Zfp367 RNAi constructs show reduction of cells in cortical plate. Knockdown of Depdc1b, Tis11b and Zfp367 caused depletion of cells from ventricular/subventricular zone.

Coronal section of the control shRNA electroporated E17.5 brain shows typical distribution of the GFP positive cells (Fig. 15). For the purpose of phenotype assessment, neurons were defined as cells outside of the VZ/SVZ having neuronal morphology. Reduction in numbers of GFP+ neurons in the cortical plate (CP) was seen in brains electroporated with Pcgf5, Ptgfrn, Tox3, Ddah1, Zfp367 and Tis11d RNAi constructs. It is unlikely that knockdown of six out of eight genes would have a similar phenotype with regard to defects in neuronal migration as manifested by decreased numbers of neurons in cortical plate and accumulation of the cells in the intermediate zone. Especially, when these genes are rather expressed at higher levels in progenitors than in neurons (Table 2).

From general lab experience, the defects in neuronal migration are often observed after electroporation of plasmids expressing shRNAs. Therefore, we attributed neuronal migration defects to the unspecific effects of the plasmid-based RNAi knockdown. We speculate that the observed migration defects could be caused by saturation of the RNAi pathway by shRNAs expressed from the electroporated plasmid, possibly acting on Dicer. Along this line, conditional deletion of Dicer has been reported to cause defects in neuronal migration (Kawase-Koga et al., 2009). For this reason genes showing neuronal migration phenotypes were not considered for further characterization.

To search for the genes that are required for self-renewal of radial glia, we focused on the genes that cause reduction in numbers of electroporated cells in the VZ/SVZ. Knockdown of Depdc1b, Tis11b and Zfp367 resulted in depletion of the cells from the VZ/SVZ (Fig.15).

The strength of the Depdc1B RNAi phenotype was highly variable between the replicate experiments. The reason for this variability is unknown and the characterization of the gene was not pursued any further.

To characterise Zfp367 RNAi phenotype in greater detail, sections were stained for Pax6 to label radial glia. Depletion of Zfp367 resulted in the reduction of the electroporated Pax6

positive radial glia from the ventricular zone (Fig. 16A). To test whether the observed phenotype is specific, we electroporated RNAi-resistant Zfp367 overexpression construct together with the shRNA plasmid. As a control, Zfp367 shRNA plasmid was co-electroporated with an empty expression vector. The RNAi phenotype could not be rescued by co-electroporation of RNAi-resistant construct, suggesting that the observed phenotype was caused by the off-target effect (Fig. 16B). Recently, Zfp367 knockout mice have been generated by KOMP (NIH-funded Knockout Mouse Project, Project ID: CSD36052). The mice are viable, fertile and do not display any abnormalities, suggesting that the loss of radial glia in Zfp367 RNAi condition is indeed a non-specific off -target effect.

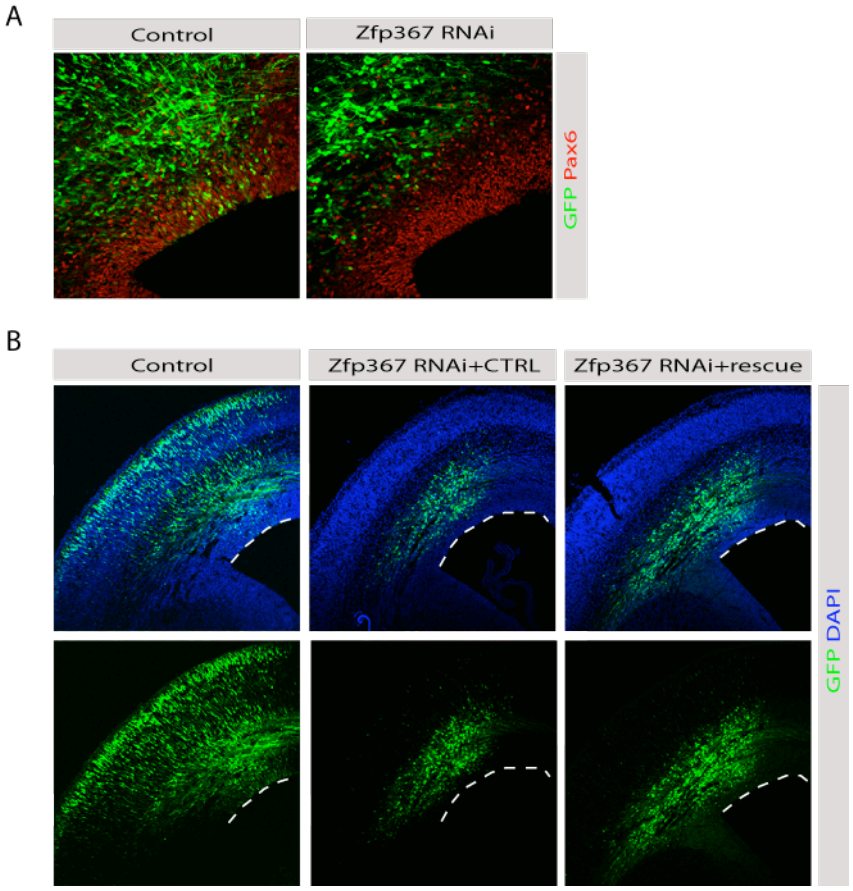


Figure 16. Zfp367 RNAi phenotype is caused by off target effects. A. Ventricular zone of E17.5 neocortex electroporated with control or Zfp367 RNAi vectors at E14.5. Zfp367 RNAi brains have reduced numbers of Pax6+ electroporated radial glia **B.** Loss of cells from ventricular zone in Zfp367 RNAi condition can't be rescued by co-electroporation of Zfp367 RNAi-resistant construct. Dashed line outlines ventricular surface.

Knockdown of Tis11b gave a very strong phenotype with complete loss of the electroporated cells from the VZ/SVZ. The GFP+ cells accumulated in intermediate zone and cortical plate without indication of strong neuronal migration defect. This suggests that Tis11b is required for maintenance of the neural progenitors. Function of Tis11b in corticogenesis will be described in the following chapter.

Discussion

We have employed transcriptome analysis of cortical progenitors and neurons to identify novel candidate genes required for self-renewal of cortical progenitors. Purification of progenitors and neurons directly from the developing neocortex allowed us to circumvent cultivation of the cells *in vitro*. We consider this very important, since the relationship between the cultured neural stem cells and neural progenitors *in vivo* is still a subject of many debates (reviewed in Conti and Cattaneo, 2010). Additionally, many aspects of neurogenesis, such as production of intermediate progenitors, regional identity and differentiation potential are not recapitulated well *in vitro*. FACS-sorting has been used for purification of neural progenitors by others as well, where authors used general markers of radial glia to isolate progenitors of the neocortex (Abramova et al., 2005; Ohtsuka et al., 2011; Pinto et al., 2008). In our strategy, we used Pax6 to drive expression of the fluorescent marker. Pax6 is specifically expressed by the progenitors of the dorsal but not ventral telencephalon. The use of this marker allows us to sort cells not only according to radial glial identity, but also according to the regional identity of the cells.

Recently, the transcriptome profiling of the lasser-microdissected ventricular zone and cortical plate has been published (Ayoub et al., 2011; Fietz et al., 2012). Although the published results are similar to our data, we identified more differentially expressed genes, using the same false discovery rates.

To identify novel regulators of neurogenesis we attempted to integrate results from the RNAi screen of self-renewal regulators in *Drosophila* neuroblasts with transcriptome analysis of murine neural progenitors. We searched for the murine orthologues of the genes that gave phenotype in the RNAi screen and are at the same time upregulated in

progenitors. Although this approach retrieved several known regulators of neurogenesis, the majority of the identified genes were involved in general control of the cell cycle.

This somewhat surprising result indicates that for many genes that share similar roles in neural development in both species, the expression patterns do not necessarily have to match as well. One possibility is that *Drosophila* larval brain neuroblasts are more similar to progenitors in an area of the brain other than the neocortex, meaning that in this case we would miss all the factors that have dual roles in establishment of the regional identity and control of the neurogenesis. Many key discoveries about control of the neurogenesis in mouse were based on previous work in lower model organisms. Indeed, our lab has successfully used transfer of knowledge between *Drosophila* and mouse to elucidate key processes controlling brain development (Postiglione et al., 2011; Schwamborn et al., 2009).

As an alternative strategy, we used literature-based search of the most differentially expressed genes. Knockdown of the first eight candidates in developing neocortex led to identification of Tis11b as a novel regulator of murine corticogenesis.

Tis11b controls self-renewal of neural progenitors

Introduction

Gene expression is regulated transcriptionally and post-transcriptionally at multiple levels. Control of mRNA stability and degradation is essential component of gene expression regulation at post-transcriptional level. Alterations in mRNA turnover have been implicated in several diseases. Deregulation of mRNA stability control has been observed in human cancer (Al-Souhibani et al., 2010; Iwanaga et al., 2011; Kanies et al., 2008). Moreover, polymorphisms in genes of the mRNA decay machinery have been associated with inflammatory diseases such as rheumatoid arthritis, underlining the importance of gene expression control at the level of mRNA stability for normal physiology (Carrick et al., 2006; Suzuki et al., 2008).

Messenger RNAs that are subjected to rapid turnover typically have adenylate- uridylate-rich elements (ARE) in their 3′-untranslated region (reviewed in Sanduja et al., 2011). Approximately 10% of the genes in human genome contain AU-rich elements in their 3′ UTR (Halees et al., 2008). The ARE motifs are recognized by a set of RNA-binding proteins that promote destabilization of these mRNAs. Several proteins have been described in mammals to bind ARE motifs and promote mRNA decay such as Auf1, KH domain RNA binding protein (KSRP), and members of the Tis11 family (Baou et al., 2009; Carballo et al., 1998; Gherzi et al., 2004; Zhang et al., 1993).

The family of Hu proteins can also bind AU-rich elements within the 3′-UTR of the mRNAs (Hinman and Lou, 2008). In contrast to the ARE-binding proteins that promote mRNA degradation, Hu proteins promote expression of the target genes by preventing degradation of the transcripts. Competitive binding to the vascular endothelia factor ARE-rich mRNA has been described between the stabilizing Hu proteins and destabilizing Tis11 proteins (Cherradi et al., 2006). This indicates that the stablility of the ARE mRNA depends on the balance between the actions of the ARE mRNA stabilizing and destabilizing factors.

Tis11 proteins are among the best-characterised ARE-mediated mRNA decay-promoting proteins. There are four members of the Tis11 family, Tis11 (Zfp36), Tis11b (Zfp36l1), Tis11d (Zfp36l2) and Zfp36l3. The last identified member of the family Zfp36l3 is murine-specific and its expression is confined to the extra-embryonic tissues, such as placenta and yolk sac (Blackshear et al., 2005). Tis11 family proteins are required for normal mouse development and health. Tis11 knockout mice are born normal, but develop a systemic inflammation. The inflammatory phenotype is caused by increased expression of the Tis11 target gene tumor necrosis factor alpha (TNF-α) (Taylor et al., 1996). Treatment with anti-TNF-α antibody cures virtually all pathologies in the Tis11 knockout mice. This indicates that although Tis11 has been shown to regulate many messenger RNAs, deregulation of the single gene TNF-α is responsible for the systemic inflammation and pathology in Tis11 knockout mice.

Tis11b knockouts have defects in chorioallanotic fusion and haematopoiesis in the yolk sac. Abnormal placental development leads to death by embryonic day 11 (Bell et al., 2006; Stumpo et al., 2004).

Tis11d is required for normal haematopoiesis. It has been shown that Tis11d knockout mice die within few weeks after birth due to an inability to maintain haematopoietic stem cells (Stumpo et al., 2009).

Tis11b and Tis11d act redundantly in T cell development. Mice with a conditional deletion of both genes develop T cell acute lymphoblastic leukemia T-ALL (Hodson et al., 2010). Microarray analysis of Tis11b/d double cKO T cells revealed upregulation of their target gene Notch1. Increased expression of Notch1 likely contributes to the onset of leukemia in the Tis11b/d double cKO mice, since murine models of T-ALL typically harbor activating mutations in Notch1 (O'Neil et al., 2006).

Tis11 family proteins interact with the core nonameric ARE motif UUAUUUAUU in the 3'UTR of mRNA via two CCCH-type zinc finger domains (Lai et al., 2002). Genome-wide analyses of the Tis11 targets have revealed that mRNAs containing shorter ARE motifs, such as AUUUA can also be degraded by Tis11 (Emmons et al., 2008; Lai et al., 2006).

Removal of the polyA-tail is the first step in ARE-mediated mRNA decay (Fig. 16). Tis11 and Tis11b have been shown to directly interact with Ccr4-Not1 deadenylation complex (Lykke-Andersen and Wagner, 2005). Additionally, Tis11 can promote the activity of the poly(A) RNAse (PARN) to degrade the polyA-tail (Lai et al., 2003). No direct interaction between PARN and Tis11 has been detected, suggesting that Tis11 acts on PARN via other proteins (Lykke-Andersen and Wagner, 2005). The deadenylation activity is optimally induced by binding of the Tis11 proteins to the two nonameric ARE motifs (Lai et al., 2005). In the presence of only one motif, the induction of the deadenylation activity is reduced.

Removal of the polyaA-tail renders mRNAs susceptible to degradation. Tis11 proteins can directly recruit and promote the activity of the exosome complex, which degrades deadenylated mRNA in 3'-5' direction. Additionally, interaction between Tis11 and deccaping enzymes Dcp1, Dcp2 and exonuclease Xrn1 promotes mRNA decapping and 5'-3' degradation (Fenger-Gron et al., 2005; Lykke-Andersen and Wagner, 2005).

Recently a novel function for Tis11b has been described in endothelial cells (Desroches-Castan et al., 2011). Cytoplasmic localization has been reported for Tis11b in different cell types (Benjamin et al., 2006; Cherradi et al., 2006). However in endothelial cells Tis11b is localized mainly in the nucleus where it has beed described to target Notch ligand Delta-like 4 (Dll4) (Desroches-Castan et al., 2011). In contrast to the well-known function of Tis11b as a mRNA destabilizer, in endothelial cells Tis11b does not act on the stablility Dll4 mRNA. Instead Tis11b, prevents recognition of polyadenylation signal, resulting in the extension of Dll4 3′-UTR (Desroches-Castan et al., 2011). Extension of the 3′-UTR promotes degradation of the Dll4 mRNA.

To summarise, Tis11 family of proteins recognize AU-rich transcripts and promote the degradation of the target mRNAs by recruitment of the multiple components of the RNA decay machinery. Additionally, Tis11b at least in endothelial cells, can downregulate gene expression via modulation of the 3′-UTR extension.

Tis11b was identified as one of the hits in our in vivo RNAi candidate screen for novel regulators of neurogenesis in the developing neocortex. No function in brain development has been described for Tis11 proteins or ARE-mediated decay. Phenotype of the Tis11b knockout mice has been characterised, but early embryonic death at E11 prevents analysis of the Tis11b function in the developing CNS. Our results from the RNAi screen indicate that Tis11b and ARE- mediated decay pathway could control neurogenesis in the developing neocortex.

Results

ARE-mediated decay genes are upregulated in neural progenitors

To learn more about the potential role of Tis11b and ARE-mediated decay in cortical development, we first examined expression of the genes that provide sequence specificity to the mRNA decay machinery, taking advantage of the transcriptome profiles of cortical progenitors and neurons of the E14.5 neocortex. RNA-binding proteins acting as mRNA destabilizers (Auf1, Khsrp, Tis11 family) were all expressed in the cortical progenitors (Fig. 17A). Tis11 was expressed at lowest (FPKM=7.23) and Auf1 (FPKM=445.81) at highest

level within the group. Tis11b and Tis11d were only two differentially expressed genes (FDR 1%) (Fig17B). Tis11b and Tis11d were expressed at higher levels in the progenitors than in neurons with Log2FC of 5.09 and 2.76, respectively. Within the core mRNA decay machinery, several members of the exosome complex (Exosc2, 3, 7, 8) and decapping enzyme Dcp2 were also expressed at significantly higher levels in progenitors than neurons (Fig. 17E, asterisks). None of the Ccr4-Not complex genes were significantly differentially expressed.

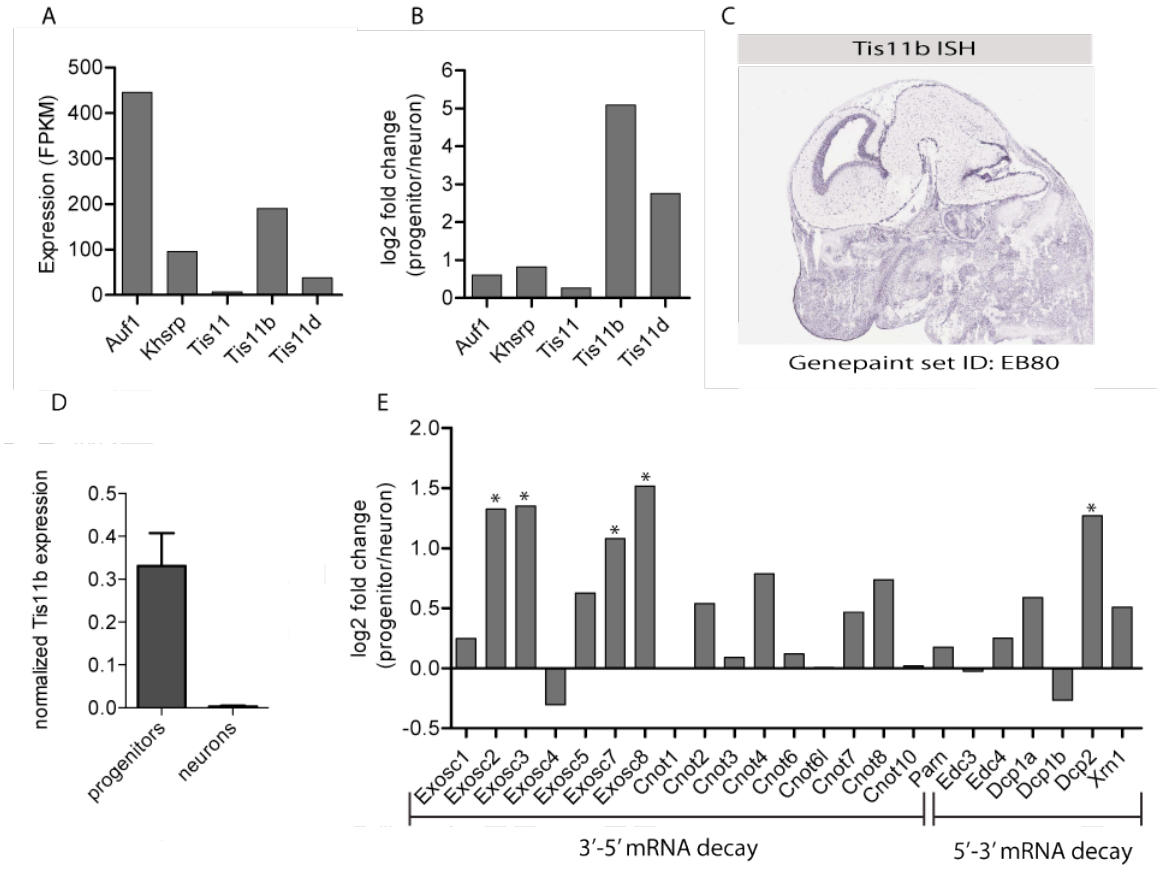


Figure 17. Genes of AU-rich mRNA decay machinery are upregulated in cortical progenitors. Expression of mRNA decay pathway genes in RNA seq of E14.5 cortical progenitors and neurons. **A.** ARE element-binding mRNA destablilizing genes are expressed in progenitors. (FPKM, fragments per kilobase per million mapped reads) **B.** Tis11b and Tis11d are significantly upregulated in progenitors (FDR 1%). **C.** In situ hybridisation of Tis11b mRNA in E14.5 brain. (Genepaint set ID: EB80, http://www.GenePaint.org) **D.** Tis11b is upregulated in sorted progenitors, as measured by qPCR. (n=3, error bars SEM). **E.** Members of the core mRNA decay markers are upregulated in progenitors. Significantly upregulated genes are marked with asterisk. (FDR 1%).

Both, Tis11b and Tis11d are significantly upregulated in progenitors compared to neurons. Since Tis11d did not show any phenotype in the in utero electroporation RNAi screen, we focused our work on the function of Tis11b in cortical neurogenesis.

To verify differential expression of the Tis11b in the RNA seq profiling, we measured levels of Tis11b mRNA in FACS-sorted neural progenitors and neurons by RT-qPCR. Expression of Tis11b mRNA was 147-fold higher in sorted progenitors than in neurons confirming the expression pattern of Tis11b in the RNA seq data (Fig. 17D). Additionally, publicly available in situ hybridization databases of developing mouse embryo show high expression of Tis11b mRNA in ventricular zone of the E14.5 embryonic neocortex (Genepaint set ID: EB80, http://www.GenePaint.org) (Fig. 17C).

Tis11b RNAi causes exit of the cells from ventricular zone

To address the role of Tis11b in neurogenesis we decided to deplete Tis11b in radial glia by RNAi. We designed short hairpin RNAs (shRNA) targeting open reading frame of the murine Tis11b mRNA. To test the efficiency of the shRNAs we transfected Tis11b shRNA plasmids together with V5-tagged Tis11b expression vector into HEK 293T cells and measured knockdown by Western blot. We generated potent Tis11b shRNA that mediated strong knockdown of Tis11b comparing to control shRNA (Fig. 18A).

To deplete Tis11b in cortical progenitors, we performed in utero electroporation of Tis11b or control non-targeting RNAi vector into radial glia of E14.5 brains. EGFP expression vector was added to the shRNA plasmids to track the electroporated cells. Distribution of the GFP positive cells in the coronal sections of the electroporated brains was analysed three days later at E17.5.

Knockdown of Tis11b caused altered distribution of the electroporated cells in the cortex compared to the control (Fig. 18B). Quantification of the GFP+ cell distribution showed significant decrease in percentage of the cells occupying VZ/SVZ in Tis11b RNAi cortices (3.6%) compared to the control RNAi (29.2%) (Fig. 18C, D). Significantly higher fraction of the EGFP+ cells was found in the intermediate zone (IZ) following Tis11b RNAi (54.3%) compared to the control shRNA electroporated cells (35.5%). The proportion of cells in the cortical plate was not significantly different between the two conditions.

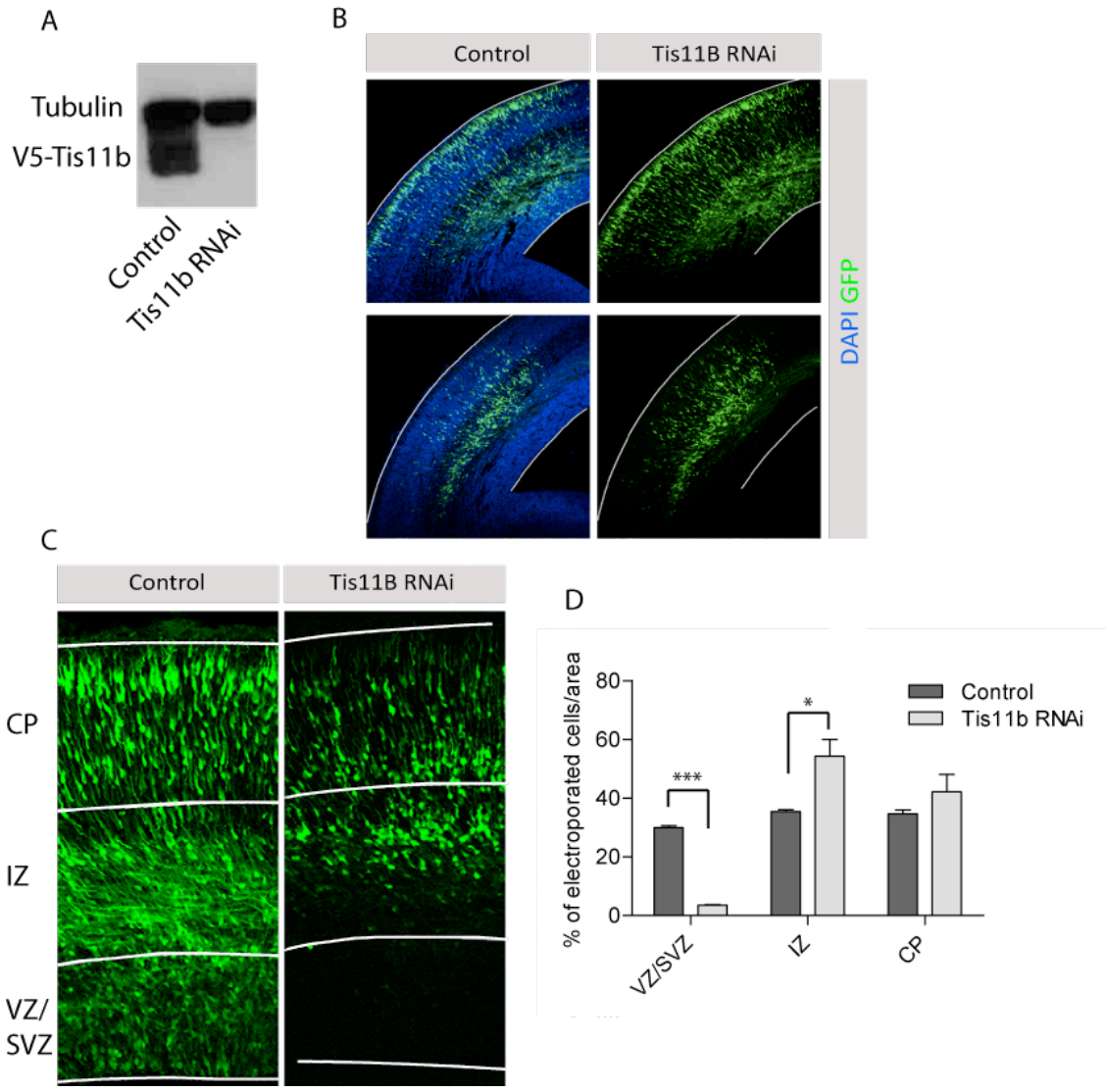


Figure 18. Knockdown of Tis11b causes depletion of cells from ventricular/subventricular zone. A. Western blot of V5-tagged Tis11b cotransfected with Tis11b RNAi into HEK 293T cells. **B.** Brains electroporated with Tis11b RNAi or control vectors at E14.5 and fixed 3 days later. Knockdown of Tis11b caused altered distribution of electroporated GFP+ cells. White line marks ventricular and pial surface. **C.** GFP+ cells are lost from VZ/SVZ in E17.5 cortices electroporated with Tis11b RNAi at E14.5. **D.** Quantification of distribution of GFP+ cells in Tis11b RNAi and control electroporated cortices. (n=3, error bars SEM, unpaired t-test, * p value<0.05 *** p value<0.001).

Depletion of the GFP+ cells from the VZ/SVZ and concomitant increase in GFP+ cells in IZ of the Tis11b RNAi electroporated cortices points towards premature differentiation of the radial glia. This result suggests that Tis11b could be required for self-renewal of cortical

progenitors. Alternatively, similar distribution of the electroporated cells could be caused by cell death of progenitors, meaning that Tis11b would be required for survival of radial glia.

Tis11b is not required for survival of neural progenitors

To distinguish between these two possibilities, we first investigated whether loss of the electroporated cells from the VZ/SVZ is caused by apoptosis. For this, we fixed E14.5 electroporated brains 2 and 2.5 days after electroporation. Before these timepoints no prominent loss of the cells from VZ/SVZ was observed. To cause such a strong depletion of the cells from the VZ/SVZ in Tis11b RNAi condition by E17.5, massive cell death should be observed before. To detect the apoptotic cells, the sections were stained for activated caspase 3. We did not observe an increase in apoptotic GFP+ cells in the VZ/SVZ at any of the stages in Tis11b RNAi brains. Occasionally, an apoptotic GFP+ cell within intermediate zone was detected in Tis11b RNAi electorporated brains (Fig. 19, arrowhead). Presence of the activated caspase 3 positive cells in the sections shows that staining worked properly. Additionally increased cell death would cause accumulation of the small GFP+ aggregates/cells with pyknoic nuclei in the ventricular zone, this was however not observed. To conclude, depletion of the electroporated cells from the VZ/SVZ following Tis11b RNAi is not caused by cell death. Thus, Tis11b is not required for survival of the cortical progenitors.

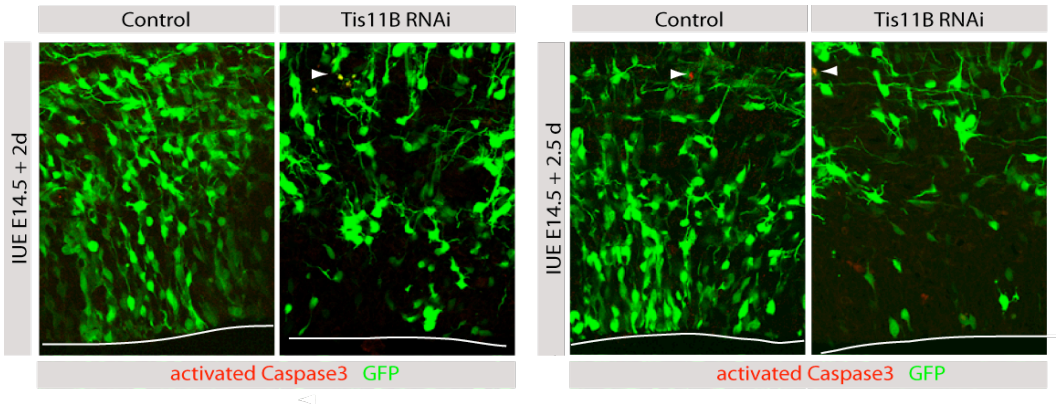


Figure 19. Tis11b knockdown does not cause cell death. Cortices electroporated at E14.5 and stained for apoptotic marker activated Caspase 3 at 2 and 2.5 d post electroporation. No increase in apoptotic electroporated cells in VZ was observed. White line outlines ventricular surface. Arrowheads show occasional apoptotic GFP+ cells in intermediate zone.

Tis11b controls maintenance of cortical progenitors

Depletion of the cells from the VZ/SVZ and lack of the cell death indicates premature differentiation. To ask how knockdown of Tis11b affects fate of the neural lineage in the developing necortex, we introduced RNAi via in utero electroporation into E14.5 embryos and analysed the identity of the GFP+ cells at E17.5. To quantify numbers of the radial glial cells, the sections were stained for Pax6, a marker of neocortical radial glia. Fraction of GFP+ radial glia was significantly lower in the VZ of Tis11b RNAi brains (0.82%), compared to the control brains (12.3%) (Fig. 20 A,E). No ectopic localization of Pax6 + GFP+ cells in Tis11b RNAi was detected, meaning that loss of Tis11b does not cause displacement and ectopic accumulation of neural progenitors.

Similarly, the numbers of Tbr2 positive intermediate progenitors were diminished in Tis11b knockdown brains. In the control, 9.5% of GFP+ cells were Tbr2 positive. Tis11b RNAi reduced numbers of intermediate progenitors to 0.55% (Fig. 20B,F).

To demonstrate that knockdown of Tis11b caused neuronal differentiation, we performed staining for Satb2, which is the marker of neurons that are born after embryonic day 14. GFP+ cells in Tis11b RNAi in the intermediate zone are largely Satb2 positive, similar to the control condition (Fig. 20C). This means that depletion of Tis11b forced neuronal differentiation, producing neurons of the correct Satb2 positive identity.

To examine the effect of Tis11b depletion on progenitor proliferation, the brains were stained for mitotic marker pH3, to quantify the mitotic index. Strikingly, mitotic GFP+ cells were never detected in any of the Tis11b RNAi cortices (Fig. 20D,G). This means that few electroporated cells positive for progenitor markers Pax6 and Tbr2 in Tis11b RNAi condition were likely progenitors committed to differentiation.

Analysis of cell fate shows dramatic reduction of cortical progenitors following depletion of Tis11b by RNAi, meaning that Tis11b is essential to maintain self-renewing potential of radial glial cells. These results could indicate that activity of the ARE-mediated decay pathway guided by Tis11b to the specific targets is required in neural progenitors, possibly to prevent upregulation of the target genes that promote neuronal differentiation.

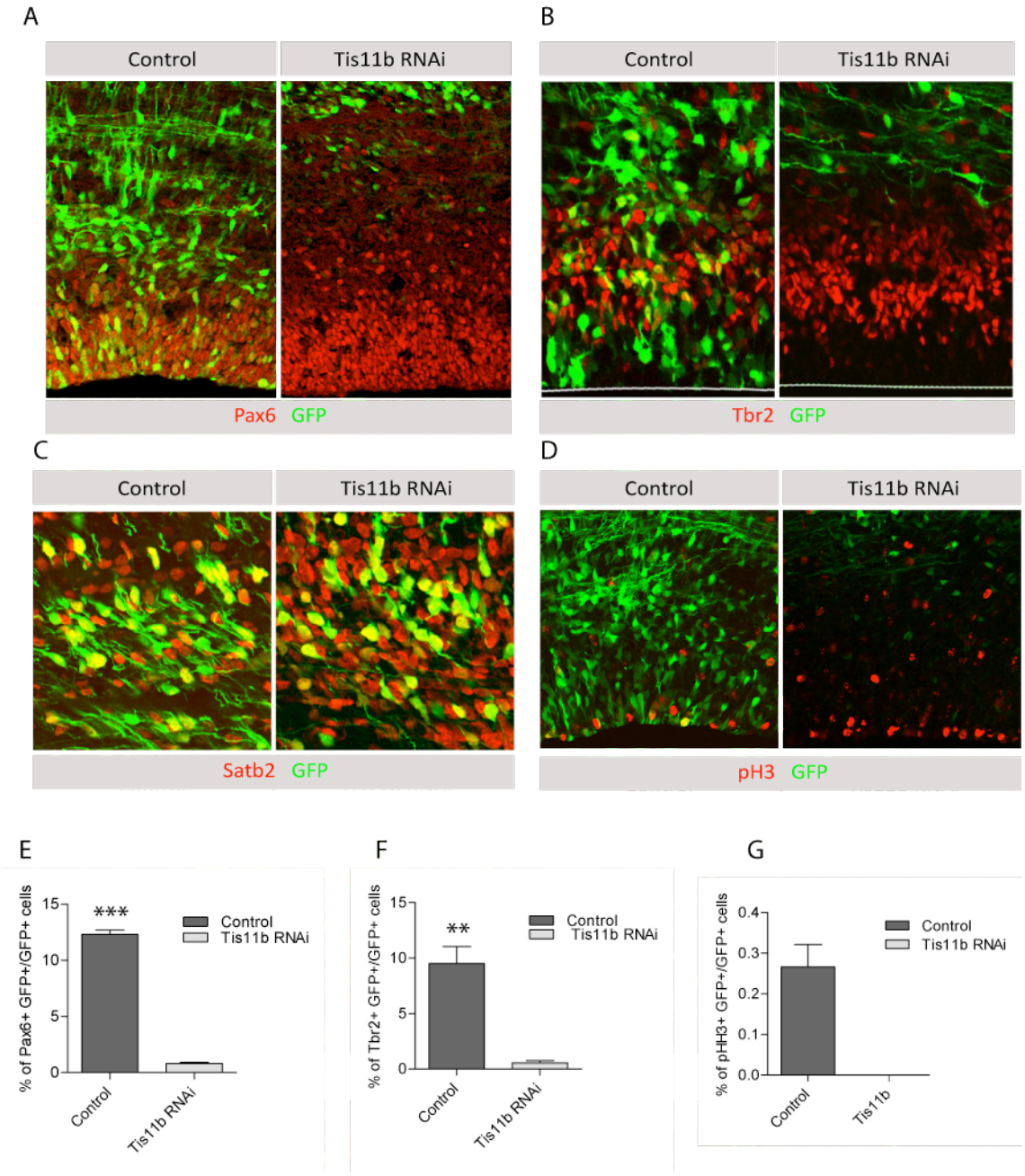


Figure 20. Tis11b RNAi leads to premature differentiation and depletion of progenitor pool. E14.5 brains electroporated with Tis11b RNAi or control vector and fixed 3 days later. Knockdown of Tis11b resulted in almost complete loss of Pax6+ radial glia **(A, E)** and Tbr2+ intermediate progenitors **(B, F)** comparing to control electroporated brains. Tis11b RNAi promotes neuronal differentiation of progenitors. Tis11b RNAi and control cells in the intermediate zone are positive for neuronal marker Satb2 **(C).** Complete loss of proliferating progenitors following Tis11b knockdown. No mitotic phospho-histone3+ GFP+ cells were observed in Tis11b RNAi electroporated brains **(D,G)**. (n=3 brains from independent electroporations, error bars SEM, unpaired t-test ** p value 0.01, *** p value 0.001)

Tis11b knockdown phenotype can be rescued by RNAi-resistant construct

RNAi is widely used to rapidly address loss of function phenotypes. The methodology inherently suffers from off-target effects, where the observed phenotype is caused by the unintended knockdown of the gene that has partial complementarity to the sequence of the RNAi. To verify that the phenotype caused by the knockdown of the Tis11b is specific, we generated a Tis11b expression vector resistant to the RNAi. The RNAi-resistant Tis11b expression construct was made by synonymous codon mutations in the binding site of the shRNA.

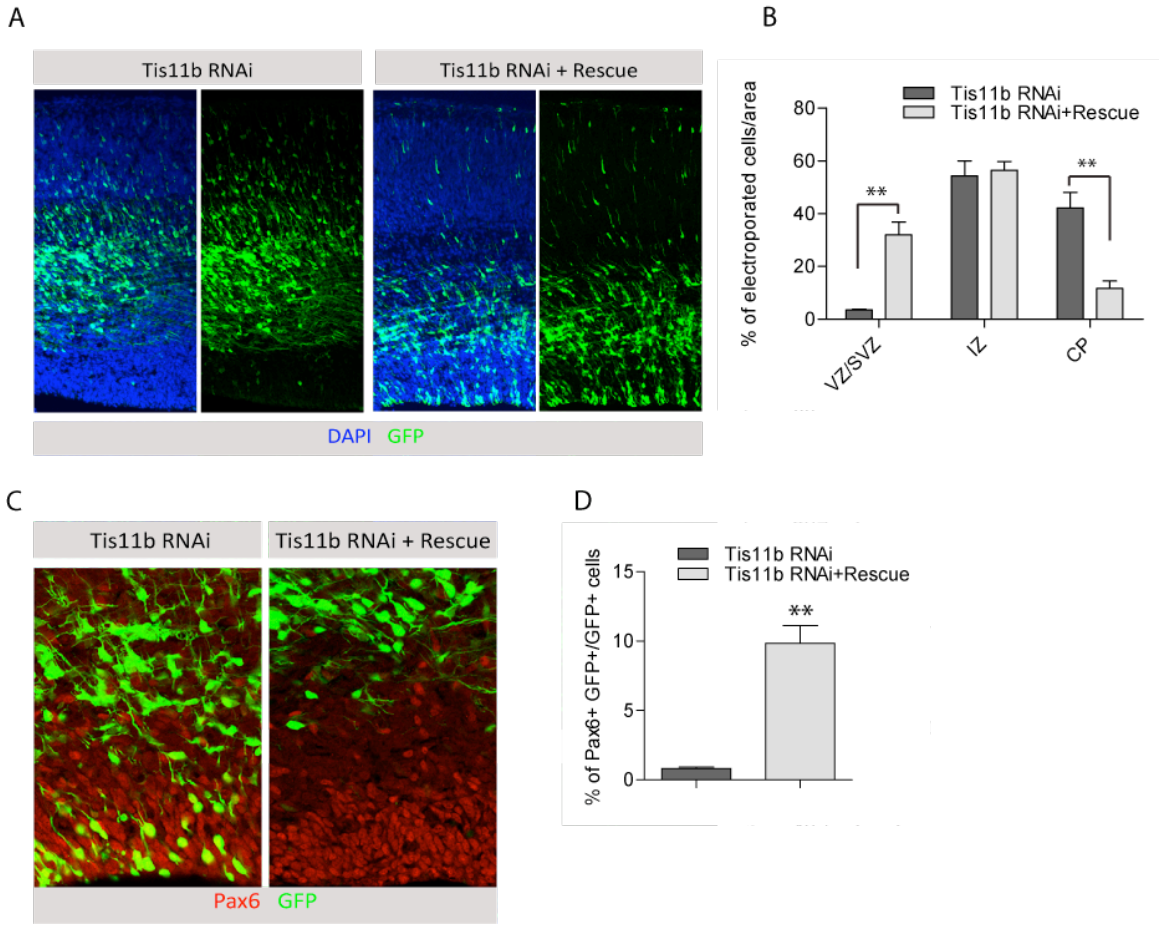


Figure 21. Tis11b RNAi phenotype can be rescued by RNAi-resistant construct. In utero electroporation at E14.5 + 3 days. **A.** Expression of RNAi-resistant Tis11b together with RNAi prevents loss of cells from the VZ/SVZ. **B.** Distribution of GFP+ cells in the electroporated cortices. **C.** Loss of Pax6+ radial glial cells in Tis11b RNAi is rescued by expression of the RNAi-resistant form of Tis11b, quantified in **D**. (n=3 brains from independent electroporations, error bars SEM, unpaired t-test, ** p value<0.01)

We performed in utero electroporation of the Tis11b RNAi together with the RNAi-resistant Tis11b vector at E14.5 and fixed the brains three days later. Distribution of the electroporated cells shows that coexpression of the RNAi-resistant Tis11b rescued depletion of the electroporated cells from the ventricular zone observed in Tis11b RNAi (Fig. 21A,B).

Next, we stained sections for Pax6 to quantify numbers of cortical radial glia. Rescue of the Tis11b RNAi restored Pax6+ radial glia in the ventricular zone of the electroporated cortices (Fig. 21C). Quantification shows 9% increase in the GFP+ Pax6+ cells in rescue condition comparing to the RNAi (Fig. 21D).

We can conclude that premature differentiation phenotype observed with in utero electroporation of Tis11b RNAi is specific as it can be rescued by coexpression of RNAi-resistant form of Tis11b.

Prediction of Tis11b targets in cortical radial glia

Mechanistically, Tis11b causes mRNA destabilization via binding to the 3′-UTR of the AU-rich messenger RNAs (reviewed in Sanduja et al., 2011). ARE-mediated decay via Tis11 proteins does not lead to complete depletion of the target mRNA, but rather to a decrease in the levels of target transcripts. We hypothesized that targets of Tis11b that contribute to the premature differentiation phenotype in Tis11b RNAi should be present in our transcriptome profile of cortical progenitors. Moreover these mRNAs should have a functional AU-rich elements in their 3′-UTR. Expression levels of such Tis11b targets would increase during neuronal differentiation. Although we have performed profiling of the cortical progenitors and neurons, our transcriptome profiles do not allow reliable identification of the genes that increase during early stages of neuronal differentiation. The reason is that the cortical plate neurons used for RNA sequencing have already downregulated many genes that transiently increase their expression in commited progenitors and early differentiating progeny such as transcription factors Neurog1, Neurog2 and Tbr2 involved in differentiation. For this reason we did not utilize information about the differential gene expression in prediction of Tis11b targets.

To identify putative targets of Tis11b we searched for the presence of ARE motifs in the 3'UTR of the genes expressed in the progenitors with FPKM<1. We selected all genes containing at least one heptameric (TATTTAT) or longer motif. By applying these criteria we retrieved 3209 genes. Presence of at least two motifs has been described to optimally target mRNA for degradation, but even mRNAs with one motif can be subjected to the destabilization by Tis11 proteins.

To identify genes involved in neuronal differentiation we performed a Gene Ontology term enrichment analysis to find genes associated with GO term neuron differentiation. In total, we identified 51 genes that contain at least one ARE motif in the 3'UTR and are annotated with GO term neuronal differentiation (Table 3).

To investigate ARE motifs in greater detail we used AREScore program that provides numerical value of potential ARE motif strength based on the number, distance and sequence context of the ARE motifs (Spasic et al., 2012). Transcriptome-wide median AREScore for mouse is 1.3. Medain AREScore of Tis11 bound mRNAs is 7.8 (Spasic et al., 2012).

The list contained several well-known factors that are involved in regulation of neuronal differentiation such as transcription factors Neurogenin2, Neurod4, Notch ligand Delta-like1, and Ndel1 (Table 3, highlighted in blue). Notch1 receptor, already demonstrated target of Tis11b (Hodson et al., 2010) is present in the list as well. Two out of these five genes, Neurogenin2 (Ngn2) and Delta-like1 (Dll1), are known to potently induce neuronal differentiation when overexpressed in radial glia (Ge et al., 2006; Kawaguchi et al., 2008). Additionally, ARE motifs of Ngn2 and Dll1are well conserved across mammalian species.

Since both Ngn2 and Dll1 contain conserved putative ARE motifs and have well-documented function in neuronal differentiation, we decided to test if they are regulated by Tis11b.

Table 3. List of genes expressed in progenitors that have ARE-motifs in 3'UTR and are annotated with GO term neuron differentiation. Genes with known function in differentiation are highlighted in blue

Gene symbol	AREScore	Gene symbol	AREScore	Gene symbol	AREScore
Onecut2	43.25	Robo2	11.1	App	3.9
Bmpr1b	27.85	Whrn	11	Brsk2	3.9
Neurod4	21.65	Rab3a	9.5	Dll1	3.9
Bcl2	21	Dst	7.95	Ablim1	3.6
Creb1	20	Neurog2	7.9	Stxbp1	3.6
Prkg1	18	Brsk1	7.1	Bmp4	2.6
Ntrk2	17.15	Rpgr	7.1	Epha2	2.6
Myo6	15.45	Sema5a	6.9	Notch3	2.6
Epha7	15.2	Notch1	6.7	Ndel1	2.3
Jag1	13.45	Wnt7a	6.7	Ptpn11	2.3
Vegfa	13.4	Efna5	5.2	Ryk	2.3
Prkci	12.9	Tgfb2	5.2	Cdh4	1.3
Abi2	12.6	Ulk2	5.2	Pak1	1
Sall3	12.6	Dvl1	5.1	Alkbh1	0
Mtpn	12.1	Cdk5r1	4.9	Bmp7	0
Sema6a	12.1	Trappc4	4.1	Sema6c	0
Epha4	11.6	Apbb2	3.9	Sema6c	0

Depletion of Tis11b upregulates expression Ngn2 and Dll1

To address whether proneural factors Neurog2 and Dll1 could be targets of Tis11b we decided to examine whether their mRNA levels change following Tis11b RNAi. To test this, we performed in utero electroporation of both cerebral hemispheres with Tis11b shRNA plasmid together with GFP vector. Non-targeting shRNA was used as a control. The dorsal pallium was dissociated into single cell suspension 24h later and electroporated GFP positive cells were FACS-sorted directly into the TRIzol for total RNA isolation. Representative FACS plots are shown in (Fig. 22A). We were able to routinely purify between 20-50 000 cells from single electroporated brain. The levels of tested genes were analysed by quantitative PCR.

To determine the knockdown efficiency we measured levels of Tis11b mRNA (Fig. 22B). In Tis11b shRNA electroporated cells, the levels Tis11b mRNA were significantly decreased compared to the control. To test whether 24h knockdown of Tis11b is sufficient to cause upregulation of Tis11b target mRNAs, we quantified Notch1, a published target that is expressed in radial glia. We detected 1.5 fold higher levels of Notch1 mRNA in Tis11b depleted cells compared to the control (Fig. 22C). Measurement of Tis11b targets at mRNA

level by qPCR after 24h knockdown can be used to identify transcripts with deregulated expression upon depletion of Tis11b.

Next, we asked if there are any changes in Ngn2 and Dll1 expression resulting from Tis11b depletion. We detected small but significant increase in mRNA levels of Ngn2 and Dll1 in Tis11b RNAi electroporated cells (Fig. 22D,E). Small increase in expression of positive control Notch1, Ngn2 and Dll1 could be caused by incomplete depletion of Tis11b 24h after electroporation. We quantified knockdown of Tis11b on mRNA level, however some protein could be still present in the electroporated cells. We attempted to analyse changes in Tis11b targets 48h post-electroporation. But at this time point the phenotype has already manifested, meaning that by sorting for GFP positive cells we would compare cell population enriched for neurons in Tis11b RNAi compared to the control. Additionally, two housekeeping genes used for normalization of the gene expression, β-Actin and Srp14, gave different results at 48h post electroporation. We do not know the exact reason for this, but the differences are not caused by the neuronal differentiation, since none of the housekeeping genes are differentially expressed between the progenitors and neurons. Analysis after 24h did not show these inconsistencies and allowed reliable measurements of gene expression. From these experiments we can conclude that depletion of Tis11b in radial glia leads to upregulation of the two proneural genes Ngn2 and Dll1 on mRNA level.

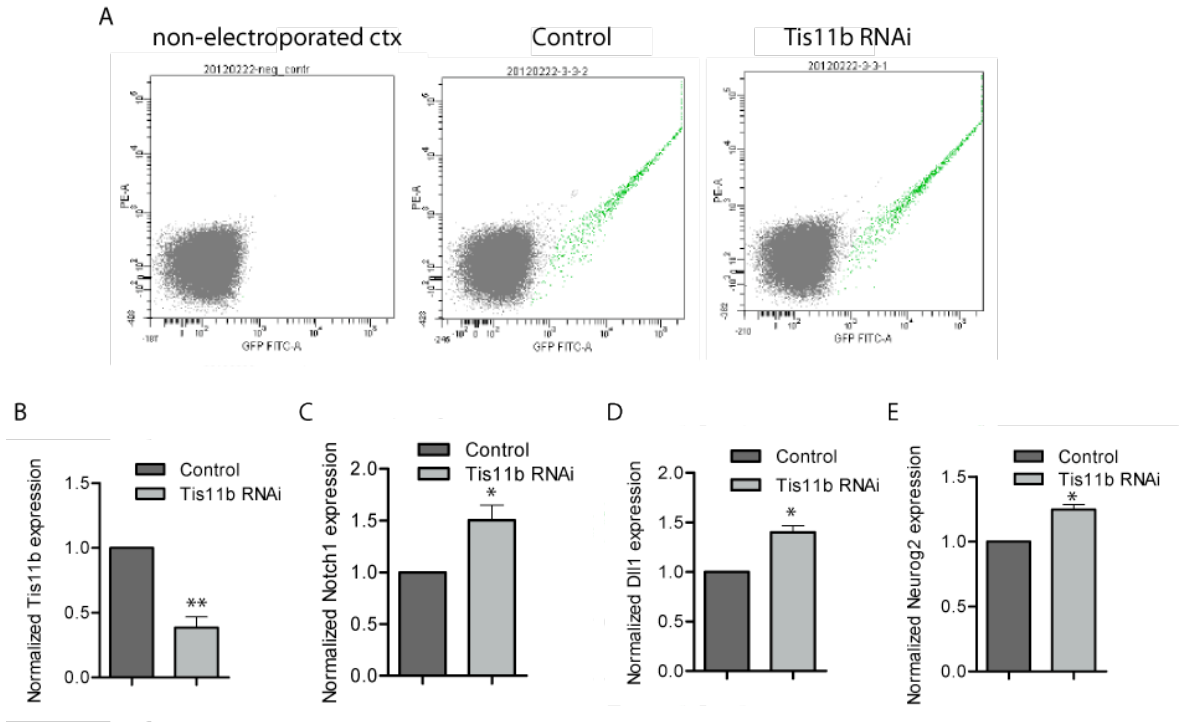


Figure 22. Tis11b knockdown upregulates expression of Ngn2 and Dll1. A. Representative FACS plots of brains electroporated at E14.5 and sorted at E15.5. **B.** Knockdown of Tis11b measured by qPCR in sorted cells. **C.** Increased mRNA levels of positive control Notch1 in Tis11b RNAi condition. **D-E.** Upregulation of Dll1 and Ngn2 expression in Tis11b depleted cells. Samples were normalised to Actin and Srp14 reference genes and to the control electroporation (n=4 brains from independent electroporations, error bars SEM, one sample t-test, * p value<0.05)

Tis11b regulates Ngn2 and Dll1 expression via their 3'UTR

Ngn2 and Dll1 are upregulated in Tis11b RNAi cells indicating that they could be the targets of Tis11b. We wanted to investigate whether Tis11b controls the expression of Ngn2 and Dll1 via their 3'UTR. To test this, we cloned full 3'UTR of the Ngn2 and Dll1 downstream of the open reading frame of the Firefly Luciferase gene in the pGL3 reporter vector modified for cloning of the 3'UTRs. Additionally, we made luciferase reporter vector with Notch1 3'UTR that served as a positive control. The reporter vectors were cotransfected with Tis11b overexpression plasmid and Renilla vector for normalization of the reporter gene activity into the NIH3T3 cells. As a control, the luciferase reporters were cotransfected with EGFP instead of Tis11b expression vector, since both proteins are similar in size and GFP does not have any effect on the activity of the reporter.

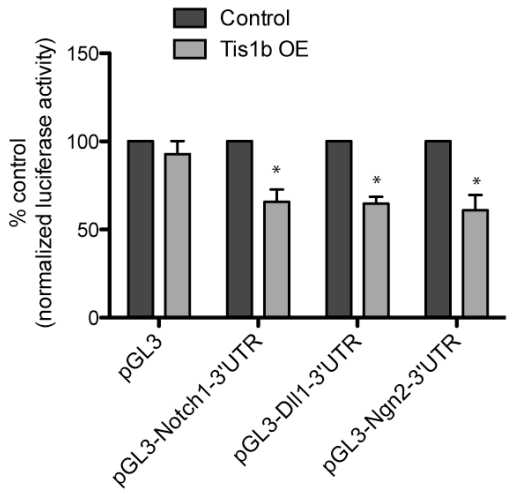


Figure 23. Ngn2 and Dll1 3′UTR is targeted by Tis11b. NIH3T3 cells transfected with pGL3 luciferase reporter containing 3′UTR of putative target genes and Tis11b expression vector or EGFP as a control. Tis11b overexpression does not have any significant effect on reporter that does not have ARE elements in 3′UTR. Activity of reporters carrying 3′UTRs of Notch1, Dll1 and Ngn2 is significantly decreased by overexpression of Tis11b. Samples were normalised to Renilla luminenscence and control transfection. (n=3 independent experiments, error bars SEM, one sample t-test p value<0.05)

Cotransfection of Tis11b did not have any significant effect on pGL3 reporter that does not have ARE motifs in the 3′UTR (Fig. 23). Luminescence of the positive control the Notch1-3′UTR reporter decreased by 35% in Tis11b overexpression condition relative to the control. Ectopic expression of Tis11b suppressed the luciferase activity of the Ngn2 and Dll1 3′UTR reporter vectors to 64% and 60% of the control, respectively. This result shows that 3′UTR of the pronerual genes Ngn2 and Dll1 can mediate suppression of the reporter activity by Tis11b protein.

These results indicate that upregulation of the Ngn2 and Dll1 following depletion of Tis11b could be regulated via their 3′UTRs.

Tis11b is a target of Notch signalling

Ngn2 and Dll1 are suppressed on transcriptional level in radial glia through Notch-activated Hes1 transcriptional repressor. Thus, Notch signalling in radial glia represses activity of the proneural genes. Considering specific expression of the Tis11b in radial glia, the cells with high Notch activity, and the fact that Tis11b controls expression of at least

two genes repressed by Notch signalling, we were wondering if of Tis11b expression itself could be regulated by Notch pathway.

To examine this, we treated cortical progenitors grown in stem cell medium with γ-secterase inhibitor DAPT to block the Notch pathway. We measured changes in Tis11b expression by quantitative PCR. We used Hes1 a direct target of Notch1/RBPj as a positive control. We detected 76% decrease in Hes1 expression after 24h treatment with DAPT, demonstrating efficient inhibition of the Notch pathway (Fig. 24B).

Inibition of Notch signalling caused 64% decrease in Tis11b mRNA levels compared to control cells treated with DMSO (Fig. 24A). This result shows that expression of Tis11b is sensitive to changes in Notch pathway activity suggesting that Tis11b could be a target of Notch signalling.

To investigate if Notch1 ICD/RBPj-k can act on Tis11b promoter, we searched for RBPj-k consensus binding sites in Tis11b promoter using MatInspector program (Genomatix). We identified two putative binding sites at position –793 and +121 (Fig. 24C). To find out if these RBPj binding sites are functional, we cloned the promoter fragment containing –793 and +121 binding sites upstream of the luciferase gene in promoterless variant of the pGL3 luciferase reporter vector.

The Tis11b promoter construct was cotransfected with increasing doses of Notch1 ICD expression vector into NIH3T3 cells. The luciferase activity was measured 24h post transfection and normalised to Renilla luminescence used as a transfection control.

Transfection of Notch1 ICD increased the Tis11b promoter activity in dose-dependent manner, comparing to the control transfected with empty vector (Fig. 24D). No significant increase in luciferase activity was observed when RBPj binding sites were deleted from the reporter plasmid, suggesting that RBPj binding sites are required to mediate transactivation of Tis11b promoter by Notch1 ICD in the luciferase reporter assay.

Taken together we have identified novel gene regulated by Notch signalling. Our results indicate Tis11b could be direct target of Notch ICD/RBPj complex.

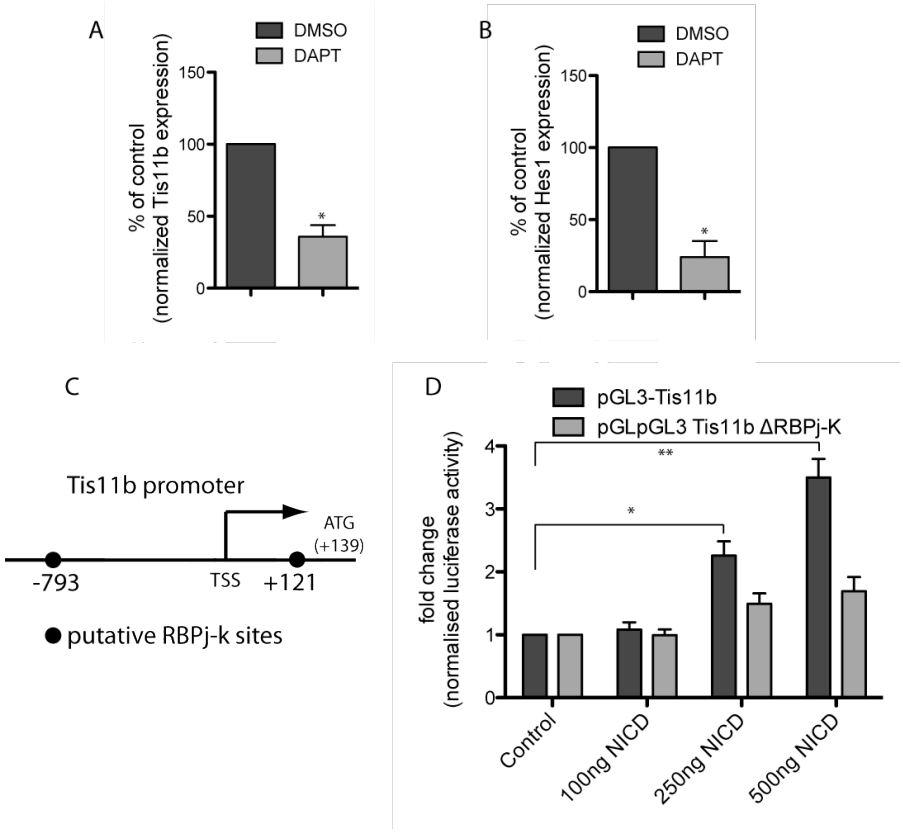


Figure 24. Tis11b is positively regulated by Notch signalling. A. Treatment of cortical progenitors with γ-secretase inhibitor DAPT to block the Notch pathway reduces mRNA levels of Tis11b as measured by qPCR. **B.** mRNA of Notch target gene Hes1 was quantified as a positive control. **C.** Scheme of murine Tis11b promoter showing putative RBPj-k binding sites. **D.** Tis11b promoter fragment containing putative RBPj-k binding sites cloned upstream of luciferase reporter shows dose-dependent increase in activity in response to increasing amounts of Notch intracellular domain. Deletion of putative RBPj-k sites prevented significant upregulation of luciferase activity.

Discussion

Gene expression is controlled at virtually every step on the path from messenger RNA to the mature protein. Regulation of mRNA stability is a critical component of gene expression control on post transcriptional level. The role of ARE-mediated decay in stem cell maintenance is not fully understood yet. Tis11d mutant mice die postnatally due to the loss of the haematopoietic stem cells (Stumpo et al., 2009). In contrast to this, deletion of Tis11b and Tis11d in T cell lineage causes accumulation of aberrant immature progenitors and T cell acute lymphoblastic leukemia (Hodson et al., 2010). Another AU-rich mRNA destablilizing protein Khsrp has been proposed to prevent differentiation of myoblast

C2C12 cells (Briata et al., 2005). But Khsrp mutant mice are fully viable and do not exhibit any defects (Lin et al., 2011). Thus, depending on the tissue, perturbations of the ARE-mediated decay can result in stem cell loss or tumor development.

Our work unravels a novel role for Tis11b in the maintenance of neural progenitors during neurogenesis in murine embryonic neocortex. Depletion of Tis11b in cortical radial glia impairs self-renewal capacity of radial glia, causing progenitor depletion by premature differentiation. Nearly all radial glia and intermediate progenitors have disappeared from the VZ/SVZ three days after in utero electroporation. Similarly, no mitotic cells were ever seen in Tis11b RNAi cortices 72h after electroporation, suggesting that few progenitors left at this stage are rather cells undergoing differentiation.

Tis11b knockout embryos show apoptosis in early neural tube, but this has been attributed to the placental defects causing death of the embryo by E11 (Stumpo et al., 2004). Severe loss of radial glia and intermediate progenitors in Tis11b knockdown brains is not caused by apoptosis, since we have not observed increase in activated caspase 3 staining or pyknoic electroporated cells.

Thus, control of AU-rich mRNAs turnover by Tis11b in cortical radial glia is essential to prevent premature exhaustion of the progenitor pool.

This suggests that genes promoting differentiation should be already expressed in the progenitors but their expression is kept under the treshold, which would promote differentiation. Indeed expression of the proneural genes has already been demonstrated in radial glia (Britz et al., 2006; Kawaguchi et al., 2008; Knuckles et al., 2012; Shimojo et al., 2008). Thus transcriptional program of cortical progenitors is possibly primed for the neuronal differentiation.

We propose that at least part of the differentiation-promoting transcriptional program in radial glia is supressed on posttranscriptional level by ARE-mediated decay via Tis11b (Fig. 25). Loss of Tis11b leads to increased expression of genes that instruct a cell to exit cell

cycle and differentiate. This would imply that neuronal differentiation could be at least in part triggered by downregulation of the ARE-mediated decay activity.

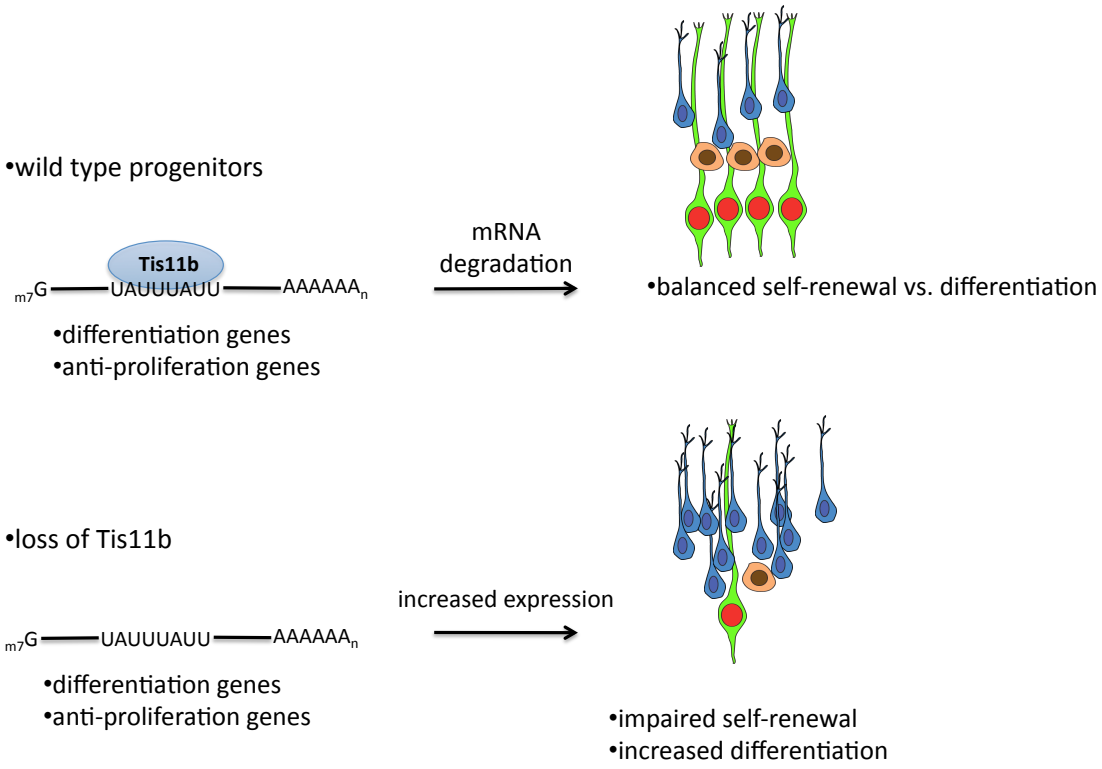


Figure 25. Proposed model how Tis11b maintains self-renewing capacity of neural progenitors. Tis11b supresses expression of genes that promote cell cycle exit and neuronal differentiation, thereby preventing depletion of progenitor pool. In Tis11b RNAi, genes inducing differentiation become expressed at levels that interfere with self-renewing capacity of radial glia and cause neuronal differentiation. Radial glia (green), intermediate progenitors (orange), neurons (blue).

Activity of the Tis11b is dependent on the phospohrylation status of the protein (Sanduja et al., 2011). Phosphorylation of Tis11b by p38 mitogen-activated protein kinase 2 reduces decay-inducing activity of the protein (Maitra et al., 2008). Interestingly, activation of the p38 mitogen-activated protein kinase (MAPK) pathway has been described during neuronal differentiation in several in vitro models (Hansen et al., 2000; Morooka and Nishida, 1998;

Oh et al., 2009). Therefore, activation of p38 MAPK pathway during neuronal differentiation could contribute to the downregulation of the ARE-mediated decay by phosphorylation of Tis11b. It still has to be demonstrated whether changes in p38 MAPK pathway and Tis11b phosphorylation status occur during neuronal differentiation of radial glia in vivo as well.

It has been shown in C2C12 myoblast cells that activity of the p38 MAPK pathway is repressed by Notch signalling. Notch pathway induces dual-specificity phosphatase MKP-1, which dephosphorylates and inhibits p38 (Kondoh et al., 2007). It is plausible that during neuronal differentiation, decrease in Notch activity could result in enhanced phosphorylation of Tis11b by p38 MAPK2 and thereby causing downregulation of Tis11b-directed ARE-mediated decay.

Tis11b is highly expressed in radial glia progenitors compared to the differentiated progeny. Additionally, several core components of the RNA decay machinery are significantly upregulated in progenitors.

Interestingly, three members of the Hu family (HuB, C, D) that act as stabilizers of the AU-rich mRNAs have well-documented neuron-specific expression (Hinman and Lou, 2008). Increased expression of the genes promoting mRNA decay in the progenitors and neuron-specific expression of mRNA stabilizers in neurons might point towards a requirement for higher turnover of the AU-rich mRNAs in radial glia compared to the differentiating progeny. It is plausible that in the radial glia, AU-rich mRNAs are subjected to the rapid degradation through action of Tis11b and possibly other decay inducing proteins. In differentiating neurons these messenger RNAs would be stabilized by decrease in Tis11b expression and activity of Hu familiy proteins. Indeed, neuron-specific Hu proteins are one of the earliest neuronal markers expressed in differentiating cells. Therefore, it would be interesting to investigate whether neuronal differentiation is accompanied by global change in turnover rates of AU-rich mRNAs.

To get insight into the potential targets of Tis11b we attempted to identify genes expressed in progenitors that have AU-rich motifs in their 3'UTRs and are associated with GO term neuronal differentiation. Among the putative Tis11b targets, Neurogenin2 and Delta-like1 can promote neuronal differentiation when expressed at high levels in radial glia (Asprer et al., 2011; Kawaguchi et al., 2008; Knuckles et al., 2012).

Using in utero electroporation combined with FACS-sorting of electroporated cells we show that knockdown of Tis11b in cortical progenitors caused upregulation of Dll1 and Ngn2 at mRNA level. Furthermore, we demonstrate, that Dll1 and Ngn2 3'UTR can mediate repression of the luciferase reporter by Tis11b in NIH3T3 cells. Our data shows that Tis11b regulates expression of Ngn2 and Dll1 in cortical radial glia. In vitro experiments suggest that this regulation is mediated via 3'UTRs of the genes.

Ngn2 and Dll1 are expressed in neural progenitors in an oscillatory manner (Shimojo et al., 2008). The oscillations are maintained by oscillatory expression of Hes1.

Additionally, Ngn2 can directly bind to the Dll1 promoter to upregulate its expression (Castro et al., 2006). Oscillatory expression of Ngn2 and Dll1 is thought to maintain Notch signalling in radial glia, to ensure proper self-renewal. Upon differentiation, Ngn2 and Dll1 levels stop oscillating and become stabilized at high levels (Kageyama et al., 2009; Shimojo et al., 2008). Short half-life of Ngn2 and Dll1 mRNAs is a prerequisite for their oscillatory expression in radial glial cells. Our data indicate that Tis11b could be the factor controlling rapid turnover of these messenger RNAs.

Question remains, whether upregulation of Dll1 and Ngn2 expression observed in Tis11b knockdown cortices contributes to the early differentiation phenotype. Increased expression of Dll1 has been shown to promote neuronal differentiation (Kawaguchi et al., 2008). Similarly, overexpression of Ngn2 in radial glia enhances neuronal differentiation and exit of the cells from ventricular zone (Ge et al., 2006; Knuckles et al., 2012; Li et al., 2012a). But it has been shown recently that strong proneural activity of the Ngn2 decreases after E14 (Li et al., 2012a), suggesting that Ngn2 is likely not a major contributor to the premature neuronal differentiation phenotype in Tis11b-depleted cortical progenitors.

To identify Tis11b targets in the developing neocortex we are planning to examine the effect of Tis11b depletion on transcriptomes of the radial glia. Since many of the transcription factors associated with the neuronal differentiation contain putative AU-rich elements in their 3'UTRs, it will be interesting to investigate whether Tis11b mediates broader repression of the transription factor or signalling networks that promote cell cycle exit and neuronal differentiation.

Notch signalling pathway is critically required for proper self- renewal of the cortical progenitors in part through Notch-mediated repression of the genes promoting differentiation. Notch signalling induces repression of the target genes via direct activation of Hes family of transcriptional repressors. Proneural factors Ngn2, Dll1, Hes6, Eomes (Tbr2), Insm1 and Neurod4 are among the genes repressed by Hes1 (Sansom et al., 2009; Shimojo et al., 2008). Hes1 represses proneural factors on transcriptional level, but mRNAs of proneural genes can be still detected in neural progenitors, leaving the possibility that they could be regulated on post-transcriptional level as well. For example, it has been recently published that Ngn2 mRNA is destabilized in neural progenitors by cleavage of the mRNA by Drosha (Knuckles et al., 2012).

Our results show that expression of Tis11b is controlled by Notch signalling. Analysis of the Tis11 promoter using luciferase reporter assay suggests that Tis11 could be direct target of Notch pathway. In near future we will perform chromatin immunoprecipitation experiments to verify binding of Notch1/RBPj-k complex to the Tis11b promoter in embryonic neocortex.

Identification of Tis11b as a novel Notch target unravels novel layer of gene expression control mediated by Notch signalling. In addition to the transcriptional repression by Hes genes, we propose that Notch pathway could regulate stability of AU-rich element containing mRNAs via activation of Tis11b expression.

Our experiments suggest that Notch singalling could promote self-renewal by upregulating Tis11b and thus ARE-mediated decay. Question remains to what extend does ARE-mediated decay pathway contribute to the progenitor maintenance governed by Notch.

Therefore it would be interesting to test whether impaired self-renewal associated with loss of Notch signalling could be at least partially rescued by overexpression of Tis11b. This could establish a direct link between the Notch signalling and turnover of AU-rich mRNAs in the regulation of the delicate balance between the self-renewal and differentiation during neurogenesis in the embryonic neocortex.

To summarise, we demonstrate that Tis11b is essential to prevent depletion of the progenitors in the developing neocortex. We propose that Tis11b is required to keep expression of certain ARE-containing genes below the levels that could impair self-renewal and induce cell cycle exit and differentiation (Fig. 25). Based on our identification of new Tis11b targets Delta-like1 and Neurogenin2 we propose a model where Notch signalling prevents upregulation of the proneural genes not only on transcriptional level via Hes transcriptional repressors but also on level of mRNA decay via upregulation of Tis11b (Fig. 26).

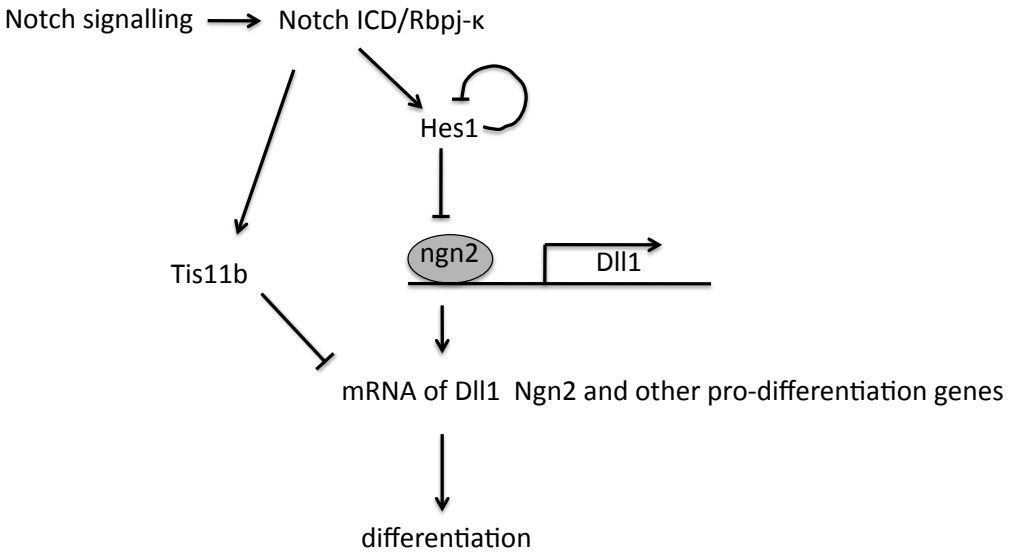


Figure 26. Proposed model integrating Tis11b mediated mRNA decay and Notch signalling in maintenance of neural progenitors. Notch signalling positively regulates expression of Tis11b to promote mRNA decay of Ngn2, Dll1 and other genes that would induce differentiation when expressed at high levels in cortical radial glia. Additionally, Tis11b could participate in oscillatory expression of Ngn2 and Dll1 by maintaining high turnover of their mRNAs.

The role of NKAP in cortical development

Introduction

The genome-wide RNAi screen of self-renewal in *Drosophila* larval brain has identified an uncharacterised gene CG6066 as a factor required for proper formation of the neuroblast lineage (Neumuller et al., 2011). Onur Kaya a colleague graduate student in the lab has described that CG6066 controls lineage progression in type II neuroblasts of the larval central brain.

Asymmetric divisions of the type II neuroblasts give rise to self-renewing neuroblast and immature intermediate neural progenitor (INP) (reviewed in Homem and Knoblich, 2012). Upon maturation, the INPs undergo multiple rounds of asymmetric cell division producing self-renewing INP and a ganglion mother cell (GMC). The GMC divides once more into two neurons. Thus, INPs represent a transit-amplifying component of the type II lineage. Loss of CG6066 results in defects in INP maturation, causing block in the lineage progression.

The CG6066 gene has a highly conserved mouse ortholog NKAP. Based on the data from *Drosophila* and the high conservation of the protein between flies and mice we decided to investigate whether NKAP could have a function in mammalian neural lineage.

NKAP is a broadly expressed nuclear protein with no known domains. It has been first identified as a factor that can activate NF-kappa B pathway (Chen et al., 2003). NKAP has been described to interact with CBF-interacting corepressor (CIR) and histone deacetylase HDAC3, both members of the Notch corepressor complex, suggesting its function as a repressor of the Notch pathway (Pajerowski et al., 2009). Conditional inactivation of the NKAP in T cells leads to a block in T cell development (Pajerowski et al., 2009). Consistent with the proposed role of NKAP as a Notch repressor, authors have observed increased expression of the Notch target genes in T cells lacking NKAP. However, it is unclear whether the phenotype can be fully attributed to the Notch signalling perturbations, raising the possibility that NKAP also acts outside the Notch pathway. Conditional inactivation of NKAP in hematopoietic stem cells leads to the proliferation defects and increased

apoptosis, ultimately resulting in failure of the hematopoietic system due to the depletion of the hematopoietic stem cells (Pajerowski et al., 2010).

Results

NKAP is essential for the development of cerebral cortex

To investigate the role of NKAP in corticogenesis we took advantage of the NKAP conditional knockout mice (kindly provided by Virginia Shapiro, University of Pennsylvania). The NKAP floxed mice were crossed with Emx1-cre to delete NKAP in progenitors of the dorsal telencephalon. Emx1-cre mice drive recombination of the floxed allele in most areas of the dorsal telencephalon (Gorski et al., 2002). Emx1-cre driven recombination can be detected by E10.5, allowing deletion of the conditional allele at the beginning of neurogenesis.

To look for possible brain defects in NKAP mutant mice, the gross cortical anatomy was examined at E18.5 a day before the birth. Comparing to control littermates, the brains of NKAP cKO mice have decreased size of the hemispheres, with apparent reduction in medial/dorsal cortex, the area where recombination of the floxed allele was driven by Emx1-cre (Fig.27A, arrow). Haematoxilin and eosin staining of the E18.5 forebrain coronal sections shows that dorsal/medial pallium is completely absent in NKAP (Fig. 27B, arrow).

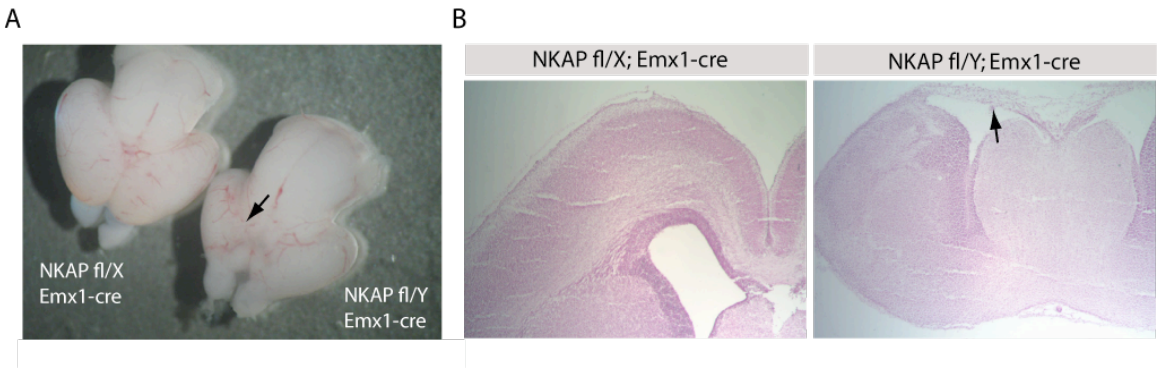
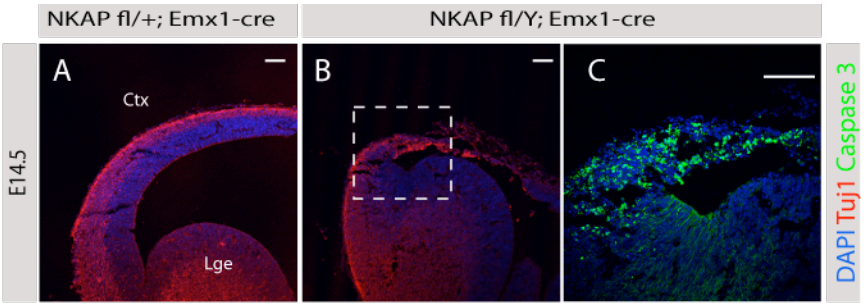
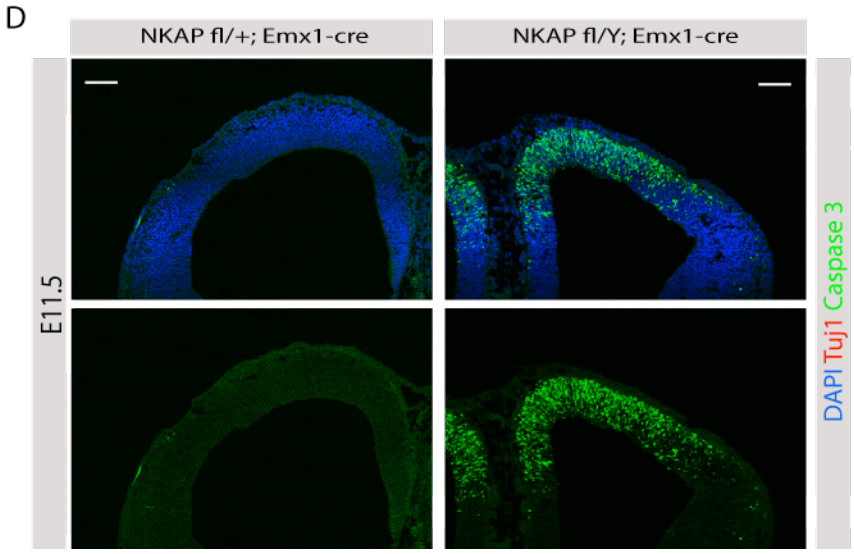


Figure 27. NKAP cKO mice lack cerebral cortex. A. NKAP floxed allele was deleted in dorsomedial pallium with Emx1-cre. E18.5 NKAP cKO mice have reduced size of cerebral cortex. **B.** Hematoxilin-Eosins staining of E18.5 coronal sections. NKAP cKO mice completely lack structures of the dorsal/medial pallium.

To investigate the cause of such dramatic phenotype, the embryos were first analysed at the peak of the neurogenesis, at embryonic day 14.5. At this stage, only fragments of the dorsal pallium were found in NKAP cKO embryos, indicating loss of the whole cortex by E14.5 (Fig. 28A,B). To test if cell death is the cause of the phenotype, we stained the brains for activated caspase 3 to detect the apoptotic cells. Remnants of the cortex were positive for activated caspase 3 showing that massive apoptosis causes loss of the cortex (Fig. 28C). To trace the onset of the cell death phenotype, the mutant brains were stained for activated caspase 3 at E11.5 and E12.5. Apoptotic cells were present already at E11.5, indicating rapid start of cell death phenotype one day after cre-mediated recombination of the floxed allele (Fig. 28D). Intact cortex, although with prominent mass of apoptotic cells was still detectable at E12.5 (Fig. 28E). This means that the collapse of the cortical structure occurs around embryonic day 13. These results show that NKAP is critical for corticogenesis, by maintaining survival of the neural cells during embryonic development.





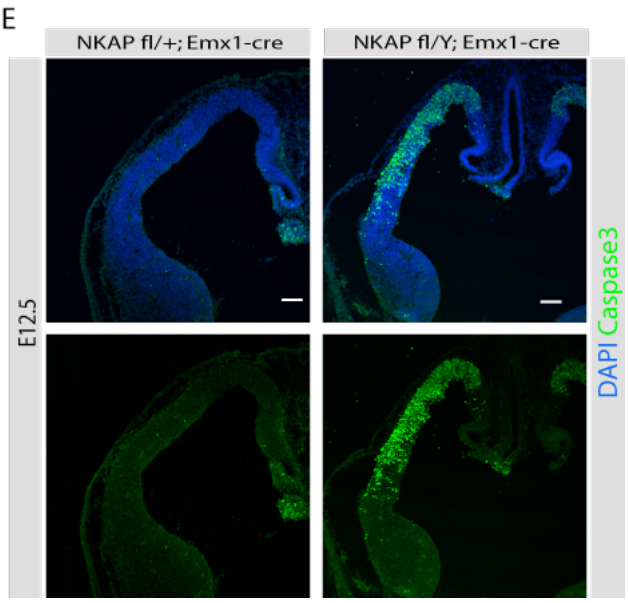


Figure 28. Deletion of NKAP causes massive apoptosis A,B. Collapse of the cortical structure in NKAP cKO mice (NKAP fl/Y; Emx1-cre) by E14.5. Neurons are labelled with Tuj1 to better visualise cortical anatomy. **C.** Fragments of the collapsed cortex contain apoptotic Caspase 3 positive cells. **D.** Cell death can be detected already at E11.5 in NKAP cKO. **E.** At E12.5 the cortex is still intact with massive apoptosis in the areas where recombination was driven by Emx1-cre.

NKAP is required for survival of neural progenitors

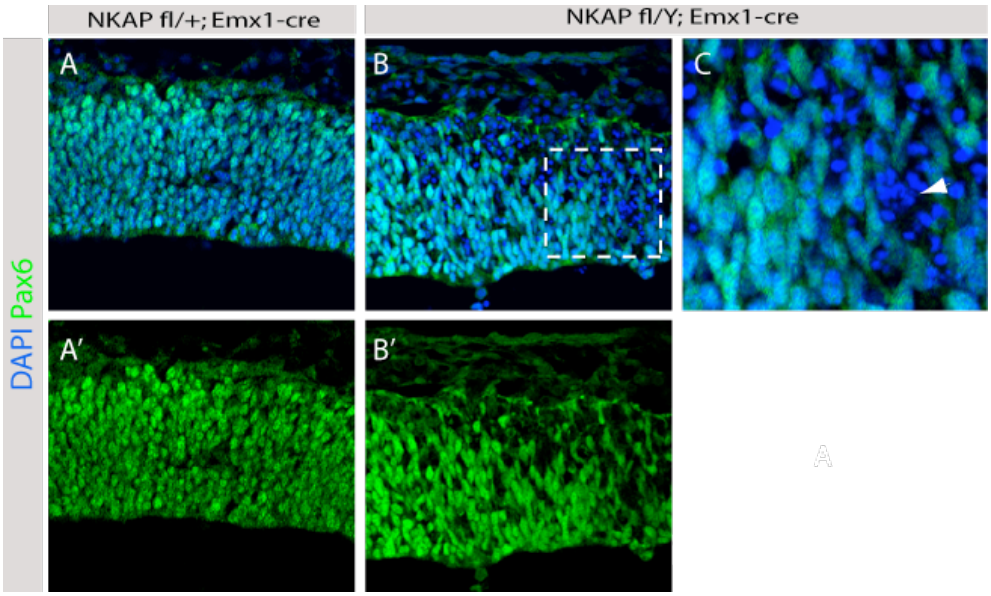
Next, we wanted to investigate which cell type is affected by apoptosis and whether any lineage defects are present in the NKAP cKO embryos. NKAP cKO have less Pax6 positive radial glia in the ventricular zone, compared to the control embryos (Fig. 29A,B). The Pax6 negative areas of the ventricular zone in NKAP cKO cortex contain pyknoic nuclei of dying cells (Fig. 29C, arrowhead). This shows that loss of Pax6+ radial glia in NKAP cKO mice is caused by prominent cell death. Therefore, NKAP is essential for viability of the radial glial cells.

The cortices of NKAP cKO mice have less Tbr2 positive intermediate progenitors compared to the control littermates (Fig. 29D). Reduction of intermediate progenitors is likely caused by massive cell death of radial glia leading to decreased progenitor pool. On the other hand, loss of NKAP could affect the cell fate decisions. Massive cell death precludes detailed analysis of the radial glial self-renewing and neurogenic divisions.

Neurons born between E10-13 form a neuronal layer located basally from the ventricular zone called preplate. In wild-type embryos, the preplate consists of a compact layer of neurons. However, in NKAP cKO the neurons are scattered across the bazal part of the ventricular zone and do not form a compact layer (Fig. 29E). Scattering of the preplate neurons is possibly caused by disruption of the ventricular zone due to the apoptosis of the radial glia, since newborn neurons migrate basally along the radial glial processes. Interestingly, numbers of neurons do not seem to be dramatically affected in comparison to the radial glia and intermediate progenitors, indicating that NKAP might not be required for the survival of postmitotic neurons.

Decreased proliferation has been observed in hematopoietic stem cells lacking NKAP, suggesting its involvement in proliferation control (Pajerowski et al., 2010). To find whether proliferation of the radial glia is affected in NKAP cKO, we stained cortices for mitotic marker, phosphorylated histone H3. Fewer dividing cells at the ventricular surface indicate proliferation defect in NKAP cKO, but this could be also result of increased cell death (Fig. 29F).

To summarise, NKAP is critical for neural development by maintaining viability of neural progenitors



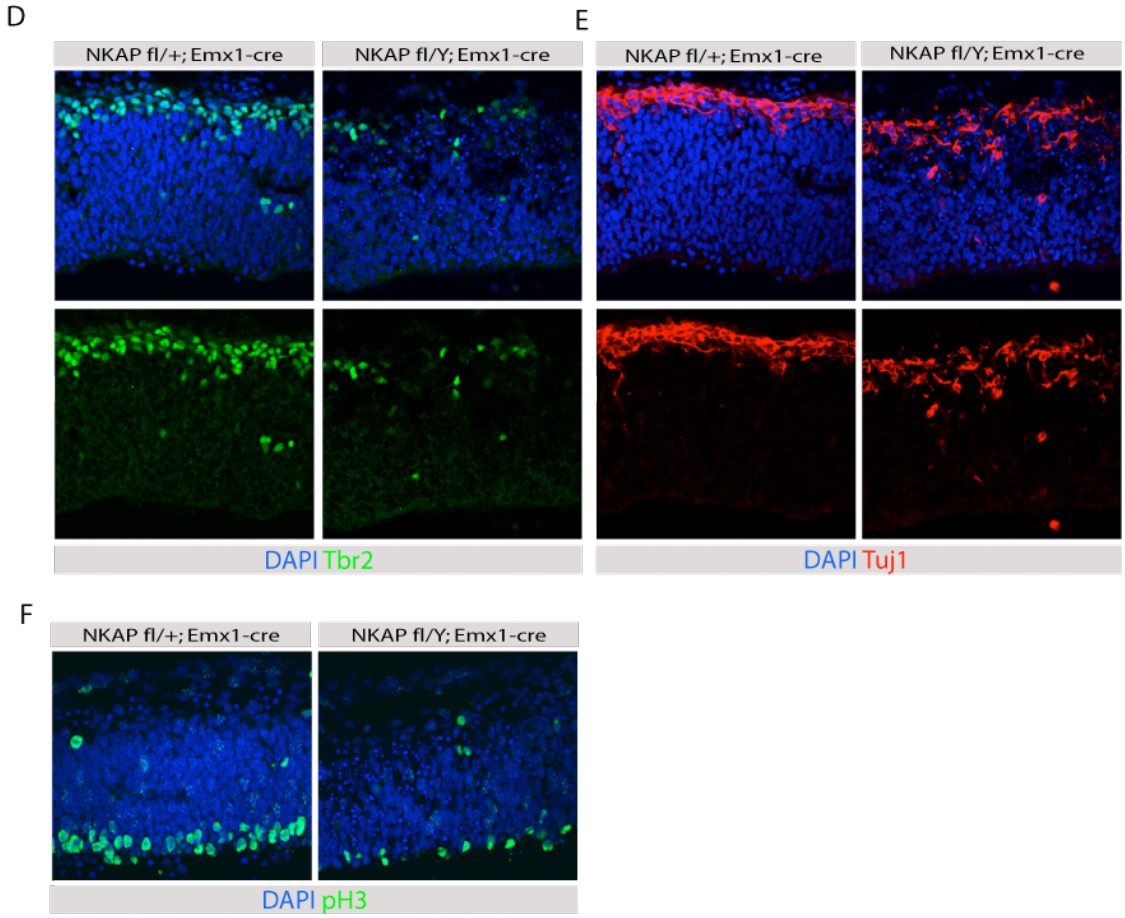


Figure 29. NKAP is required for survival of cortical progenitors. Coronal sections of E12.5 NKAP cKO cortex. **A-C.** Decreased numbers of Pax6+ radial glia in NKAP cKO. **C.** Pyknoic nuclei in ventricular zone show that NKAP is required for survival of radial glia. **D.** Numbers of Tbr2+ intermediate progenitors are reduced in NKAP cKO. **E.** Neurons in NKAP cKO are dispersed and do not form a compact layer of preplate neurons comparing to the control. **F.** NKAP cKO have reducd numbers of phospho-histone H3+ mitotic cells.

Discussion

Conditional deletion of the NKAP in the developing forebrain with Emx1-cre leads to decreased brain size caused by the absence of the whole neocortex. The neocortex disappears by embryonic day 14 as a result of the massive cell death. Conditional deletion of the NKAP in adult hematopoietic stem cells causes increased apoptosis, leading to complete loss of the hematopoiesis and death of the animal (Pajerowski et al., 2010).

However, NKAP is unlikely to be a gene generally required for cell survival, because inactivation of NKAP in T cell lineage nas no effect on the development of the γδ T cells (Pajerowski et al., 2009). Additionally, numbers of neurons in the NKAP cKO cortices do not appear to be drastically reduced compared to radial glia and intermediate progenitors, suggesting that NKAP could be essential for survival in only certain cell types. To investigate whether NKAP is required for survival of postmitotic neurons in greater detail, we plan to cross the NKAP floxed animals with mice expressing Cre from neuron-specific promoter such as Synapsin1-cre. If NKAP is not essential for neuronal survival, brains with normal anatomy should be recovered from NKAP cKO mice.

Radial glia are the primary cell type affected by the cell death in NKAP cKO brains. The reason for such massive apoptosis has not been elucidated yet. NKAP has been characterised as part of the Notch repressor complex in T cells (Pajerowski et al., 2009). But whether increased Notch activity in the neural progenitors can lead to apoptosis is controversial at the moment.

Yang et al., 2004 has observed prominent apoptosis in radial glia of the neocortex where Notch pathway had been activated by conditional overexpression of Notch1 intracellular domain (ICD). Apoptosis in these mice was caused by increased expression of p53 gene and conditional deletion of p53 in Notch1 ICD overexpression background rescued the cell death phenotype (Yang et al., 2004). In contrast to this, another study did not observe any increase in apoptosis resulting from the overexpression of the Notch1 ICD (Li et al., 2012b). Discrepancy between these two results could be caused by different approaches to overexpress Notch1 ICD. Yang et al., 2004 introduced 10-20 copies of the transgene expressed from the strong CAG promoter into the mouse genome. Li et al., 2012b generated mice that carry one copy of the transgene in ROSA26 locus expressing lower levels of Notch1 ICD that Yang 2004. Therefore different levels of the Notch activity in these two experimental setups might explain discrepancies regarding the cell death phenotype.

In mice overexpressing Notch1 ICD generated by Li et al., 2012b roughly 2.5-fold increase in mRNA levels of Notch target gene Hes1 has been detected. In NKAP cKO T cell lineage

20-80 fold increase in Hes1 expression has been measured compared to the wild type cells (Pajerowski et al., 2009).

Therefore it is possible that strong activation of the Notch target genes in NKAP cKO radial glia progenitors could contribute to the massive apoptosis consistent with the results of Yang et al., 2004. Extend of the Notch overactivation in NKAP cKO brains will be subject of further experiments.

To investigate whether Notch over-activation contributes to the cell death and loss of the cortex in NKAP mutant brains, it could be tested whether inhibition of Notch signalling in NKAP mutant background can at least partially rescue the cell death phenotype. If proven true, this would demonstrate that controlling levels of Notch signalling is essential not only to maintain the proper balance between self-renewal and differentiation, but also to ensure survial of radial glia.

Decreased proliferation evidenced by less pH3+ cells, could be also consistent with the role of NKAP as repressor of Notch target genes. It has been demonstrated that high levels of Hes1 impede cell cycle progression in neural progenitors (Shimojo et al., 2008). Therefore derepression of Notch target genes causing high levels of Hes1 could cause decreased proliferation observed in NKAP cKO mutant cortices.

To summarise, we identified essential role for NAKP in preventing apoptosis of neural progenitors in developing neocortex. Our further work will focus on elucidating the relationship between Notch signalling and survival of cortical progenitors in NKAP cKO mice.

MATERIALS AND METHODS

Animals

All mice were housed at IMP-IMBA Animal Facility under SPF conditions. Experimental procedures were approved by local authorities. In utero electroporations were performed under Experiment Licence: BMWF-66.015/0020-II/10b/2008.

For timed matings, the noon of vaginal plug was designated as embryonic day E0.5. NKAP floxed mice were characterised in (Pajerowski et al., 2009). To generate NKAP cKO mutants, floxed heterozygous females (fl/X) were crossed with Emx1-cre males. DTy54 mice expressing GFP under control of human Pax6 promoter were made by Tyas et al., 2006. Embryos for FACS sorting were obtained by breeding DTy54 homozygous males to C57BL6/J females.

In utero electroporation

Pregnant dams were anesthetized by intraperitoneal injection of Ketasol/Xylasol. Uterine horns were exposed via midline incision. For RNAi experiments, lateral ventricles of E14.5 embryos were injected with1-2 µl of 2 µg/µl of plasmid DNA containing 1.8 µg/µl of RNAi vector and 0.2 µg/µl EGFP expression plasmid. To rescue RNAi phenotypes we used mixture of 1 µg/µl of RNAi plasmid, 1 µg/µl of rescue construct and 0.2 µg/µl of EGFP expression vector.

Lateral ventricles were electroporated with tweezer type electrodes and BTX square wave electroporator (Harvard Aparatus) using following settings: 35V, 50ms pulse, 950ms pause, 5 pulses. After the procedure, abdomen was sutured with 4/0 surgical silk (Braun). Mice were kept under surveillance. Subjects usually recovered well from the procedure. Individuals exhibiting pronounced signs of discomfort were euthanized by cervical dislocation.

Immunohistochemistry

Embryonic brains were fixed in 4% (w/v) paraformaldehyde (PBS) for 30 min at room temperature followed by overnight fixation at 4°C. Fixed brains were cryoprotected in 30% (w/v) Sucrose (PBS) for 24h at 4°C, incubated with 1:1 mix of 30% sucrose (PBS) and OCT medium for 1 hour, embedded in O.C.T Compound (Finetek, Sakura) and cryosectioned at 18-20 um.

For staining, sections were rinsed in PBS, permeabilized in 0.3% (v/v) Triton-x (PBS) for 25 min, postfixed in 4% PFA for 5 min, blocked in 5% FBS, 5% BSA, 0.1% Triton-x blocking

solution and incubated with primary antibody overnight at 4°C. Sections were incubated with secondary antibodies (Molecular probes, Invitrogen) at RT for 2h. Nuclei were counterstained with 2ug/ml DAPI (PBS) for 15 min, rinsed in PBS and mounted in Fluorescence mounting medium (DAKO). Used antibodies: anti-cleaved caspase3 (rabbit, Cell Signalling) 1:100, anti-GFP (chicken, Abcam) 1:1500, anti-Ki67 (mouse, BD Biosciences) 1:100, anti-L1cam (rat, R&D Systems) 1:20, Nestin (mouse, BD Biosciences) 1:500, anti-Pax6 (rabbit, Covance) 1:300, anti-pH3 (rabbit, Chemicon) 1:400, anti-Prominin-1 (rabbit, Abcam) 1:250, anti-Satb2 (rabbit, Abcam) 1:100, anti-Sox2 (Chemicon), anti-Tbr2 (rabbit, Abcam) 1:300, anti-Tbr2 (chicken, Chemicon) 1:100, anti-Tuj1 (mouse, Sigma) 1:1000.

FACS

Dorsal pallium of E14.5 DTy54 heterozygous embryos was dissected in ice-cold DMEM/F12. Tissues were dissociated for 15 min in Accutase (Sigma-Aldrich) at 37°C and cell suspension was passed through 40μm cell strainer (BD Biosciences). The cells were incubated with PE-conjugated anti-Prominin 1 (mouse, eBioscience) 1:2000 and APC-conjugated anti-L1cam (rat, R&D Systems) 1:15 for 15 min on ice and washed three-times with DMEM/F12. For FACS, the cells were resuspended in DMEM/12 (without phenol red) supplemented with B27 and N2. Identical medium was used for collection of sorted cells. Approximately 10 000 sorted cells were used for stainings to determine purity of the sorts. The rest was used for total RNA isolation.

Preparation of cDNA library for Solexa sequencing

Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions. 1ul of GlycoBlue precipitant (Ambion, Life Technologies) was added to the RNA precipitations. RNA was dissolved in DEPC-treated water, incubated at 55°C for 5 min and cooled on ice. Polyadenylated RNA was extracted using two rounds purification with oligo(dT) beads (Invitrogen) according to the manual. Contamination with ribosomal RNAs was determined with Agilent 600 Pico Kit (Agilent).

Fragmentation of the mRNA was performed with RNA Fragmentation Reagent (Ambion) in total volume of 100µl at 70°C for 3 min. The samples were cooled on ice and purified with RNeasy columns from RNeasy Plus Mini Kit (Qiagen). Briefly, 100µl of fragmented RNA was mixed with 350µl RLT Plus buffer and 250µl 100% EtOH and applied to the column. The columns were washed twice with 500µl RPE buffer and RNA was eluted in 30µl DEPC-treated water.

For first strand synthesis, 25.5µl RNA was mixed with 1.5µl (300 ng/µl) random hexamers, 3µl 10mM dNTP mix, incubated at 65°C for 5 min and cooled on ice. Samples were mixed with 6µl 10x First Strand buffer, 12µl 25mM MgCl2, 6ul 0.1M DTT, 3µl RNaseOUT and 3µl Superscript III (200U/µl). Samples were incubated at 25°C for 10 min, 50°C for 50 min, 85°C for 5 min and cooled to 4°C. The samples were cleaned with Sephadex G-50 mini Quick Spin Columns (Roche) to remove the nucleotides according to manual.

Second strand sequencing was done as follows: purified cDNA was mixed with 1.5µl (300 ng/µl) random hexamers, 42µl 5x Second Strand Buffer, 0.42µl 100mM dATP, 0.42µl 100mM dGTP, 0.42µl 100mM dCTP, 0.42µl 100mM dUTP, 5.6 µl (50U/µl) DNA Pol I, 1.4µl (10 U/µl) DNA ligase and 1.4µl (2U/µl) RNaseH. Reactions were incubated at 16°C for 2h and subsequently purified with MinElute Reaction Cleanup Kit (Qiagen). cDNA library was quantified with Quant-iT PicoGreen dsDNA assay (Invitrogen). The samples were sequenced by IMP/IMBA Genomics facility using Solexa GAIIx or HiSeq2000 machines generating 76bp paired-end reads.

Processing of RNA seq data

The insert statistics of the strand specific paired-end reads was estimated by aligning to the genome with a maximum allowed insert size of 1000 and calculation of the mean insert length and standard deviation. The reads were aligned uniquely with TopHat (v1.2.0) (Trapnell et al., 2009) against the Mus musculus genome (mm9). Additionally, a gene model was provided as GTF (Ensembl 61). Reads of rRNAs are masked in downstream analysis. Aligned reads in valid pairs were subjected to FPKM estimation with Cufflinks (Roberts et al., 2011; Trapnell et al., 2010). In this step, bias detection and correction was

performed. Furthermore, only those fragments compatible with Ensembl annotation (61) were allowed and counted towards the number of mapped hits used in the FPKM denominator. Furthermore, the aligned reads were counted with HTSeq and the polyA containing transcripts were subjected to differential expression analysis with DESeq (Anders and Huber, 2010).

Luciferase assays

NIH3T3 cells were grown in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal bovine serum, L-Glutamine and Penicillin/Streptomycin. Transfections were performed in 24-well plates with TurboFect reagent (Fermentas) according to user's instructions. For 3'UTR assay, the cells were cotransfected with 250 ng pGL3 sensor, 5 ng Renilla-TK, 30 ng pCDNA-Tis11b or EGFP-N1 and 220ng pBluescript. Luciferase activity was measured 48h post-transfection using Dual Luciferase System (Promega).

For Tis11b promoter experiments, the cells were trasnfected with 500 ng pGL3 Tis11b promoter reporter vector + 7.5 ng Renilla TK and increasing amounts of Notch1 ICD. Luciferase activity was measured 24h post-transfection.

CONTRIBUTIONS

DTy54 mice were generated by laboratory of David Price (University of Edinburgh). NKAP floxed mice were made by laboratory of Virginia Smith Shapiro (University of Pennsylvania). Solexa sequencing was performed by IMP/IMBA Genomics Facility. Thomas Burkard performed bioinformatic analysis of the transcriptome data and wrote 'Processing of RNA seq data' part in Materials and Methods. Modified pGL3 vector for cloning and analysis of 3'UTR was made by Andrea Salva-Giro.

REFERENCES

Abramova, N., Charniga, C., Goderie, S.K., and Temple, S. (2005). Stage-specific changes in gene expression in acutely isolated mouse CNS progenitor cells. Dev Biol *283*, 269-281.

Al-Souhibani, N., Al-Ahmadi, W., Hesketh, J.E., Blackshear, P.J., and Khabar, K.S. (2010). The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes. Oncogene *29*, 4205-4215.

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol *11*, R106.

Anthony, T.E., Klein, C., Fishell, G., and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron *41*, 881-890.

Arai, Y., Pulvers, J.N., Haffner, C., Schilling, B., Nusslein, I., Calegari, F., and Huttner, W.B. (2011). Neural stem and progenitor cells shorten S-phase on commitment to neuron production. Nat Commun *2*, 154.

Arnold, S.J., Huang, G.J., Cheung, A.F., Era, T., Nishikawa, S., Bikoff, E.K., Molnar, Z., Robertson, E.J., and Groszer, M. (2008). The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone. Genes Dev *22*, 2479-2484.

Asprer, J.S., Lee, B., Wu, C.S., Vadakkan, T., Dickinson, M.E., Lu, H.C., and Lee, S.K. (2011). LMO4 functions as a co-activator of neurogenin 2 in the developing cortex. Development *138*, 2823-2832.

Attardo, A., Calegari, F., Haubensak, W., Wilsch-Brauninger, M., and Huttner, W.B. (2008). Live imaging at the onset of cortical neurogenesis reveals differential appearance of the neuronal phenotype in apical versus basal progenitor progeny. PLoS One *3*, e2388.

Ayoub, A.E., Oh, S., Xie, Y., Leng, J., Cotney, J., Dominguez, M.H., Noonan, J.P., and Rakic, P. (2011). Transcriptional programs in transient embryonic zones of the cerebral

cortex defined by high-resolution mRNA sequencing. Proc Natl Acad Sci U S A *108*, 14950-14955.

Baala, L., Briault, S., Etchevers, H.C., Laumonnier, F., Natiq, A., Amiel, J., Boddaert, N., Picard, C., Sbiti, A., Asermouh, A.*, et al.* (2007). Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis. Nat Genet *39*, 454-456.

Baou, M., Jewell, A., and Murphy, J.J. (2009). TIS11 family proteins and their roles in posttranscriptional gene regulation. J Biomed Biotechnol *2009*, 634520.

Bell, S.E., Sanchez, M.J., Spasic-Boskovic, O., Santalucia, T., Gambardella, L., Burton, G.J., Murphy, J.J., Norton, J.D., Clark, A.R., and Turner, M. (2006). The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression. Dev Dyn *235*, 3144-3155.

Benjamin, D., Schmidlin, M., Min, L., Gross, B., and Moroni, C. (2006). BRF1 protein turnover and mRNA decay activity are regulated by protein kinase B at the same phosphorylation sites. Mol Cell Biol *26*, 9497-9507.

Blackshear, P.J., Phillips, R.S., Ghosh, S., Ramos, S.B., Richfield, E.K., and Lai, W.S. (2005). Zfp36l3, a rodent X chromosome gene encoding a placenta-specific member of the Tristetraprolin family of CCCH tandem zinc finger proteins. Biol Reprod *73*, 297-307.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol *7*, 678-689.

Briata, P., Forcales, S.V., Ponassi, M., Corte, G., Chen, C.Y., Karin, M., Puri, P.L., and Gherzi, R. (2005). p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts. Mol Cell *20*, 891-903.

Britz, O., Mattar, P., Nguyen, L., Langevin, L.M., Zimmer, C., Alam, S., Guillemot, F., and Schuurmans, C. (2006). A role for proneural genes in the maturation of cortical progenitor cells. Cereb Cortex *16 Suppl 1*, i138-151.

Bultje, R.S., Castaneda-Castellanos, D.R., Jan, L.Y., Jan, Y.N., Kriegstein, A.R., and Shi, S.H. (2009). Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. Neuron *63*, 189-202.

Calegari, F., Haubensak, W., Haffner, C., and Huttner, W.B. (2005). Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. J Neurosci *25*, 6533-6538.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science *281*, 1001-1005.

Carrick, D.M., Chulada, P., Donn, R., Fabris, M., McNicholl, J., Whitworth, W., and Blackshear, P.J. (2006). Genetic variations in ZFP36 and their possible relationship to autoimmune diseases. J Autoimmun *26*, 182-196.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Gottgens, B., *et al.* (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev Cell *11*, 831-844.

Ceci, M.L., Pedraza, M., and de Carlos, J.A. (2012). The embryonic septum and ventral pallium, new sources of olfactory cortex cells. PLoS One *7*, e44716.

Chambers, C.B., Peng, Y., Nguyen, H., Gaiano, N., Fishell, G., and Nye, J.S. (2001). Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. Development *128*, 689-702.

Chen, D., Li, Z., Yang, Q., Zhang, J., Zhai, Z., and Shu, H.B. (2003). Identification of a nuclear protein that promotes NF-kappaB activation. Biochem Biophys Res Commun *310*, 720-724.

Cherradi, N., Lejczak, C., Desroches-Castan, A., and Feige, J.J. (2006). Antagonistic functions of tetradecanoyl phorbol acetate-inducible-sequence 11b and HuR in the

hormonal regulation of vascular endothelial growth factor messenger ribonucleic acid stability by adrenocorticotropin. Mol Endocrinol *20*, 916-930.

Conti, L., and Cattaneo, E. (2010). Neural stem cell systems: physiological players or in vitro entities? Nat Rev Neurosci.

Costa, M.R., Bucholz, O., Schroeder, T., and Gotz, M. (2009). Late origin of glia-restricted progenitors in the developing mouse cerebral cortex. Cereb Cortex *19 Suppl 1*, i135-143.

Costa, M.R., Wen, G., Lepier, A., Schroeder, T., and Gotz, M. (2008). Par-complex proteins promote proliferative progenitor divisions in the developing mouse cerebral cortex. Development *135*, 11-22.

Culican, S.M., Baumrind, N.L., Yamamoto, M., and Pearlman, A.L. (1990). Cortical radial glia: identification in tissue culture and evidence for their transformation to astrocytes. J Neurosci *10*, 684-692.

Del Bene, F., Wehman, A.M., Link, B.A., and Baier, H. (2008). Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. Cell *134*, 1055-1065.

Desai, A.R., and McConnell, S.K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development *127*, 2863-2872.

Desroches-Castan, A., Cherradi, N., Feige, J.J., and Ciais, D. (2011). A novel function of Tis11b/BRF1 as a regulator of Dll4 mRNA 3'-end processing. Mol Biol Cell *22*, 3625-3633.

Emmons, J., Townley-Tilson, W.H., Deleault, K.M., Skinner, S.J., Gross, R.H., Whitfield, M.L., and Brooks, S.A. (2008). Identification of TTP mRNA targets in human dendritic cells reveals TTP as a critical regulator of dendritic cell maturation. RNA *14*, 888-902.

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T., and Hevner, R.F. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci *25*, 247-251.

Farkas, L.M., Haffner, C., Giger, T., Khaitovich, P., Nowick, K., Birchmeier, C., Paabo, S., and Huttner, W.B. (2008). Insulinoma-associated 1 has a panneurogenic role and promotes the generation and expansion of basal progenitors in the developing mouse neocortex. Neuron *60*, 40-55.

Fenger-Gron, M., Fillman, C., Norrild, B., and Lykke-Andersen, J. (2005). Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. Mol Cell *20*, 905-915.

Fietz, S.A., Kelava, I., Vogt, J., Wilsch-Brauninger, M., Stenzel, D., Fish, J.L., Corbeil, D., Riehn, A., Distler, W., Nitsch, R.*, et al.* (2010). OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. Nat Neurosci *13*, 690-699.

Fietz, S.A., Lachmann, R., Brandl, H., Kircher, M., Samusik, N., Schroder, R., Lakshmanaperumal, N., Henry, I., Vogt, J., Riehn, A.*, et al.* (2012). Transcriptomes of germinal zones of human and mouse fetal neocortex suggest a role of extracellular matrix in progenitor self-renewal. Proc Natl Acad Sci U S A *109*, 11836-11841.

Franco, S.J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S.R., Ramos, C., and Muller, U. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. Science *337*, 746-749.

Frantz, G.D., and McConnell, S.K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron *17*, 55-61.

Gal, J.S., Morozov, Y.M., Ayoub, A.E., Chatterjee, M., Rakic, P., and Haydar, T.F. (2006). Molecular and morphological heterogeneity of neural precursors in the mouse neocortical proliferative zones. J Neurosci *26*, 1045-1056.

Garcia-Moreno, F., Lopez-Mascaraque, L., and De Carlos, J.A. (2007). Origins and migratory routes of murine Cajal-Retzius cells. J Comp Neurol *500*, 419-432.

Gaspard, N., Bouschet, T., Hourez, R., Dimidschstein, J., Naeije, G., van den Ameele, J., Espuny-Camacho, I., Herpoel, A., Passante, L., Schiffmann, S.N., *et al.* (2008). An intrinsic mechanism of corticogenesis from embryonic stem cells. Nature *455*, 351-357.

Ge, W., He, F., Kim, K.J., Blanchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J.I., Martinowich, K., *et al.* (2006). Coupling of cell migration with neurogenesis by proneural bHLH factors. Proc Natl Acad Sci U S A *103*, 1319-1324.

Georgala, P.A., Carr, C.B., and Price, D.J. (2011). The role of Pax6 in forebrain development. Dev Neurobiol *71*, 690-709.

Gherzi, R., Lee, K.Y., Briata, P., Wegmuller, D., Moroni, C., Karin, M., and Chen, C.Y. (2004). A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. Mol Cell *14*, 571-583.

Gorski, J.A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J.L., and Jones, K.R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. J Neurosci *22*, 6309-6314.

Gotz, M., and Huttner, W.B. (2005). The cell biology of neurogenesis. Nat Rev Mol Cell Biol *6*, 777-788.

Guillemot, F. (2005). Cellular and molecular control of neurogenesis in the mammalian telencephalon. Curr Opin Cell Biol *17*, 639-647.

Gupta, A., Tsai, L.H., and Wynshaw-Boris, A. (2002). Life is a journey: a genetic look at neocortical development. Nat Rev Genet *3*, 342-355.

Halees, A.S., El-Badrawi, R., and Khabar, K.S. (2008). ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. Nucleic Acids Res *36*, D137-140.

Hansen, D.V., Lui, J.H., Parker, P.R., and Kriegstein, A.R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature *464*, 554-561.

Hansen, T.O., Rehfeld, J.F., and Nielsen, F.C. (2000). Cyclic AMP-induced neuronal differentiation via activation of p38 mitogen-activated protein kinase. J Neurochem *75*, 1870-1877.

Harrison, S.J., Nishinakamura, R., Jones, K.R., and Monaghan, A.P. (2012). Sall1 regulates cortical neurogenesis and laminar fate specification in mice: implications for neural abnormalities in Townes-Brocks syndrome. Dis Model Mech *5*, 351-365.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development *131*, 5539-5550.

Haubensak, W., Attardo, A., Denk, W., and Huttner, W.B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. Proc Natl Acad Sci U S A *101*, 3196-3201.

Hernandez-Miranda, L.R., Parnavelas, J.G., and Chiara, F. (2010). Molecules and mechanisms involved in the generation and migration of cortical interneurons. ASN Neuro *2*, e00031.

Hinman, M.N., and Lou, H. (2008). Diverse molecular functions of Hu proteins. Cell Mol Life Sci *65*, 3168-3181.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A.J., Nye, J.S., Conlon, R.A., Mak, T.W., Bernstein, A., and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev *16*, 846-858.

Hodson, D.J., Janas, M.L., Galloway, A., Bell, S.E., Andrews, S., Li, C.M., Pannell, R., Siebel, C.W., MacDonald, H.R., De Keersmaecker, K.*, et al.* (2010). Deletion of the RNA-binding proteins ZFP36L1 and ZFP36L2 leads to perturbed thymic development and T lymphoblastic leukemia. Nat Immunol *11*, 717-724.

Homem, C.C., and Knoblich, J.A. (2012). Drosophila neuroblasts: a model for stem cell biology. Development *139*, 4297-4310.

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res *37*, 1-13.

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc *4*, 44-57.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J Neurosci *30*, 3489-3498.

Imayoshi, I., Shimogori, T., Ohtsuka, T., and Kageyama, R. (2008). Hes genes and neurogenin regulate non-neural versus neural fate specification in the dorsal telencephalic midline. Development *135*, 2531-2541.

Itoh, M., Kim, C.H., Palardy, G., Oda, T., Jiang, Y.J., Maust, D., Yeo, S.Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L.*, et al.* (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev Cell *4*, 67-82.

Iwanaga, E., Nanri, T., Mitsuya, H., and Asou, N. (2011). Mutation in the RNA binding protein TIS11D/ZFP36L2 is associated with the pathogenesis of acute leukemia. Int J Oncol *38*, 25-31.

Kageyama, R., Ohtsuka, T., and Kobayashi, T. (2008). Roles of Hes genes in neural development. Dev Growth Differ *50 Suppl 1*, S97-103.

Kageyama, R., Ohtsuka, T., Shimojo, H., and Imayoshi, I. (2009). Dynamic regulation of Notch signaling in neural progenitor cells. Curr Opin Cell Biol *21*, 733-740.

Kanies, C.L., Smith, J.J., Kis, C., Schmidt, C., Levy, S., Khabar, K.S., Morrow, J., Deane, N., Dixon, D.A., and Beauchamp, R.D. (2008). Oncogenic Ras and transforming growth

factor-beta synergistically regulate AU-rich element-containing mRNAs during epithelial to mesenchymal transition. Mol Cancer Res *6*, 1124-1136.

Kawaguchi, D., Yoshimatsu, T., Hozumi, K., and Gotoh, Y. (2008). Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon. Development *135*, 3849-3858.

Kawase-Koga, Y., Otaegi, G., and Sun, T. (2009). Different timings of Dicer deletion affect neurogenesis and gliogenesis in the developing mouse central nervous system. Dev Dyn *238*, 2800-2812.

Knoblich, J.A. (2008). Mechanisms of asymmetric stem cell division. Cell *132*, 583-597.

Knuckles, P., Vogt, M.A., Lugert, S., Milo, M., Chong, M.M., Hautbergue, G.M., Wilson, S.A., Littman, D.R., and Taylor, V. (2012). Drosha regulates neurogenesis by controlling Neurogenin 2 expression independent of microRNAs. Nat Neurosci *15*, 962-969.

Kondoh, K., Sunadome, K., and Nishida, E. (2007). Notch signaling suppresses p38 MAPK activity via induction of MKP-1 in myogenesis. J Biol Chem *282*, 3058-3065.

Konno, D., Shioi, G., Shitamukai, A., Mori, A., Kiyonari, H., Miyata, T., and Matsuzaki, F. (2008). Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. Nat Cell Biol *10*, 93-101.

Koo, B.K., Lim, H.S., Song, R., Yoon, M.J., Yoon, K.J., Moon, J.S., Kim, Y.W., Kwon, M.C., Yoo, K.W., Kong, M.P.*, et al.* (2005). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. Development *132*, 3459-3470.

Kopan, R., and Ilagan, M.X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell *137*, 216-233.

Kowalczyk, T., Pontious, A., Englund, C., Daza, R.A., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttner, W.B., and Hevner, R.F. (2009). Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. Cereb Cortex *19*, 2439-2450.

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci *32*, 149-184.

Lai, W.S., Carrick, D.M., and Blackshear, P.J. (2005). Influence of nonameric AU-rich tristetraprolin-binding sites on mRNA deadenylation and turnover. J Biol Chem *280*, 34365-34377.

Lai, W.S., Kennington, E.A., and Blackshear, P.J. (2002). Interactions of CCCH zinc finger proteins with mRNA: non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs. J Biol Chem *277*, 9606-9613.

Lai, W.S., Kennington, E.A., and Blackshear, P.J. (2003). Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. Mol Cell Biol *23*, 3798-3812.

Lai, W.S., Parker, J.S., Grissom, S.F., Stumpo, D.J., and Blackshear, P.J. (2006). Novel mRNA targets for tristetraprolin (TTP) identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts. Mol Cell Biol *26*, 9196-9208.

Latasa, M.J., Cisneros, E., and Frade, J.M. (2009). Cell cycle control of Notch signaling and the functional regionalization of the neuroepithelium during vertebrate neurogenesis. Int J Dev Biol *53*, 895-908.

Leone, D.P., Srinivasan, K., Chen, B., Alcamo, E., and McConnell, S.K. (2008). The determination of projection neuron identity in the developing cerebral cortex. Curr Opin Neurobiol *18*, 28-35.

Li, S., Mattar, P., Zinyk, D., Singh, K., Chaturvedi, C.P., Kovach, C., Dixit, R., Kurrasch, D.M., Ma, Y.C., Chan, J.A.*, et al.* (2012a). GSK3 Temporally Regulates Neurogenin 2 Proneural Activity in the Neocortex. J Neurosci *32*, 7791-7805.

Li, Y., Hibbs, M.A., Gard, A.L., Shylo, N.A., and Yun, K. (2012b). Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. Stem Cells *30*, 741-752.

Lin, W.J., Zheng, X., Lin, C.C., Tsao, J., Zhu, X., Cody, J.J., Coleman, J.M., Gherzi, R., Luo, M., Townes, T.M., *et al.* (2011). Posttranscriptional control of type I interferon genes by KSRP in the innate immune response against viral infection. Mol Cell Biol *31*, 3196-3207.

Lui, J.H., Hansen, D.V., and Kriegstein, A.R. (2011). Development and evolution of the human neocortex. Cell *146*, 18-36.

Lykke-Andersen, J., and Wagner, E. (2005). Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. Genes Dev *19*, 351-361.

Maitra, S., Chou, C.F., Luber, C.A., Lee, K.Y., Mann, M., and Chen, C.Y. (2008). The AU-rich element mRNA decay-promoting activity of BRF1 is regulated by mitogen-activated protein kinase-activated protein kinase 2. RNA *14*, 950-959.

Malatesta, P., Hartfuss, E., and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development *127*, 5253-5263.

Manabe, N., Hirai, S., Imai, F., Nakanishi, H., Takai, Y., and Ohno, S. (2002). Association of ASIP/mPAR-3 with adherens junctions of mouse neuroepithelial cells. Dev Dyn *225*, 61-69.

Meloty-Kapella, L., Shergill, B., Kuon, J., Botvinick, E., and Weinmaster, G. (2012). Notch ligand endocytosis generates mechanical pulling force dependent on dynamin, epsins, and actin. Dev Cell *22*, 1299-1312.

Merot, Y., Retaux, S., and Heng, J.I. (2009). Molecular mechanisms of projection neuron production and maturation in the developing cerebral cortex. Semin Cell Dev Biol *20*, 726-734.

Miller, F.D., and Gauthier, A.S. (2007). Timing is everything: making neurons versus glia in the developing cortex. Neuron *54*, 357-369.

Miro, X., Zhou, X., Boretius, S., Michaelis, T., Kubisch, C., Alvarez-Bolado, G., and Gruss, P. (2009). Haploinsufficiency of the murine polycomb gene Suz12 results in diverse malformations of the brain and neural tube. Dis Model Mech *2*, 412-418.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727-741.

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development *131*, 3133-3145.

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A., and Gaiano, N. (2007). Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature *449*, 351-355.

Molyneaux, B.J., Arlotta, P., Menezes, J.R., and Macklis, J.D. (2007). Neuronal subtype specification in the cerebral cortex. Nat Rev Neurosci *8*, 427-437.

Morooka, T., and Nishida, E. (1998). Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J Biol Chem *273*, 24285-24288.

Namihira, M., Kohyama, J., Semi, K., Sanosaka, T., Deneen, B., Taga, T., and Nakashima, K. (2009). Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. Dev Cell *16*, 245-255.

Neumuller, R.A., Richter, C., Fischer, A., Novatchkova, M., Neumuller, K.G., and Knoblich, J.A. (2011). Genome-wide analysis of self-renewal in Drosophila neural stem cells by transgenic RNAi. Cell Stem Cell *8*, 580-593.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714-720.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A.R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci *7*, 136-144.

O'Neil, J., Calvo, J., McKenna, K., Krishnamoorthy, V., Aster, J.C., Bassing, C.H., Alt, F.W., Kelliher, M., and Look, A.T. (2006). Activating Notch1 mutations in mouse models of T-ALL. Blood *107*, 781-785.

Oh, J.E., Bae, G.U., Yang, Y.J., Yi, M.J., Lee, H.J., Kim, B.G., Krauss, R.S., and Kang, J.S. (2009). Cdo promotes neuronal differentiation via activation of the p38 mitogen-activated protein kinase pathway. FASEB J *23*, 2088-2099.

Ohtsuka, T., Shimojo, H., Matsunaga, M., Watanabe, N., Kometani, K., Minato, N., and Kageyama, R. (2011). Gene expression profiling of neural stem cells and identification of regulators of neural differentiation during cortical development. Stem Cells *29*, 1817-1828.

Pajerowski, A.G., Nguyen, C., Aghajanian, H., Shapiro, M.J., and Shapiro, V.S. (2009). NKAP is a transcriptional repressor of notch signaling and is required for T cell development. Immunity *30*, 696-707.

Pajerowski, A.G., Shapiro, M.J., Gwin, K., Sundsbak, R., Nelson-Holte, M., Medina, K., and Shapiro, V.S. (2010). Adult hematopoietic stem cells require NKAP for maintenance and survival. Blood *116*, 2684-2693.

Pinto, L., Mader, M.T., Irmler, M., Gentilini, M., Santoni, F., Drechsel, D., Blum, R., Stahl, R., Bulfone, A., Malatesta, P.*, et al.* (2008). Prospective isolation of functionally distinct radial glial subtypes--lineage and transcriptome analysis. Mol Cell Neurosci *38*, 15-42.

Pollard, S.M., Conti, L., Sun, Y., Goffredo, D., and Smith, A. (2006). Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex *16 Suppl 1*, i112-120.

Postiglione, M.P., Juschke, C., Xie, Y., Haas, G.A., Charalambous, C., and Knoblich, J.A. (2011). Mouse inscuteable induces apical-basal spindle orientation to facilitate intermediate progenitor generation in the developing neocortex. Neuron *72*, 269-284.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol *145*, 61-83.

Rasin, M.R., Gazula, V.R., Breunig, J.J., Kwan, K.Y., Johnson, M.B., Liu-Chen, S., Li, H.S., Jan, L.Y., Jan, Y.N., Rakic, P.*, et al.* (2007). Numb and Numbl are required for maintenance of cadherin-based adhesion and polarity of neural progenitors. Nat Neurosci *10*, 819-827.

Roberts, A., Trapnell, C., Donaghey, J., Rinn, J.L., and Pachter, L. (2011). Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biol *12*, R22.

Sahara, S., and O'Leary, D.D. (2009). Fgf10 regulates transition period of cortical stem cell differentiation to radial glia controlling generation of neurons and basal progenitors. Neuron *63*, 48-62.

Sanduja, S., Blanco, F.F., and Dixon, D.A. (2011). The roles of TTP and BRF proteins in regulated mRNA decay. Wiley Interdiscip Rev RNA *2*, 42-57.

Sansom, S.N., Griffiths, D.S., Faedo, A., Kleinjan, D.J., Ruan, Y., Smith, J., van Heyningen, V., Rubenstein, J.L., and Livesey, F.J. (2009). The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet *5*, e1000511.

Sauer, F.C. (1935). Mitosis in the neural tube. J Copm Neurol *62*, 377–405.

Schenk, J., Wilsch-Brauninger, M., Calegari, F., and Huttner, W.B. (2009). Myosin II is required for interkinetic nuclear migration of neural progenitors. Proc Natl Acad Sci U S A *106*, 16487-16492.

Schwamborn, J.C., Berezikov, E., and Knoblich, J.A. (2009). The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell *136*, 913-925.

Sessa, A., Mao, C.A., Hadjantonakis, A.K., Klein, W.H., and Broccoli, V. (2008). Tbr2 directs conversion of radial glia into basal precursors and guides neuronal amplification by indirect neurogenesis in the developing neocortex. Neuron *60*, 56-69.

Shen, Q., Wang, Y., Dimos, J.T., Fasano, C.A., Phoenix, T.N., Lemischka, I.R., Ivanova, N.B., Stifani, S., Morrisey, E.E., and Temple, S. (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat Neurosci *9*, 743-751.

Shimogori, T., and Ogawa, M. (2008). Gene application with in utero electroporation in mouse embryonic brain. Dev Growth Differ *50*, 499-506.

Shimojo, H., Ohtsuka, T., and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron *58*, 52-64.

Shimojo, H., Ohtsuka, T., and Kageyama, R. (2011
). Dynamic expression of notch signaling genes in neural stem/progenitor cells. Front Neurosci *5*, 78.

Shitamukai, A., Konno, D., and Matsuzaki, F. (2011). Oblique radial glial divisions in the developing mouse neocortex induce self-renewing progenitors outside the germinal zone that resemble primate outer subventricular zone progenitors. J Neurosci *31*, 3683-3695.

Shitamukai, A., and Matsuzaki, F. (2012). Control of asymmetric cell division of mammalian neural progenitors. Dev Growth Differ *54*, 277-286.

Siegenthaler, J.A., Ashique, A.M., Zarbalis, K., Patterson, K.P., Hecht, J.H., Kane, M.A., Folias, A.E., Choe, Y., May, S.R., Kume, T.*, et al.* (2009). Retinoic acid from the meninges regulates cortical neuron generation. Cell *139*, 597-609.

Slater, J.L., Landman, K.A., Hughes, B.D., Shen, Q., and Temple, S. (2009). Cell lineage tree models of neurogenesis. J Theor Biol *256*, 164-179.

Spasic, M., Friedel, C.C., Schott, J., Kreth, J., Leppek, K., Hofmann, S., Ozgur, S., and Stoecklin, G. (2012). Genome-Wide Assessment of AU-Rich Elements by the AREScore Algorithm. PLoS Genet *8*, e1002433.

Stancik, E.K., Navarro-Quiroga, I., Sellke, R., and Haydar, T.F. (2010). Heterogeneity in ventricular zone neural precursors contributes to neuronal fate diversity in the postnatal neocortex. J Neurosci *30*, 7028-7036.

Stumpo, D.J., Broxmeyer, H.E., Ward, T., Cooper, S., Hangoc, G., Chung, Y.J., Shelley, W.C., Richfield, E.K., Ray, M.K., Yoder, M.C., *et al.* (2009). Targeted disruption of Zfp36l2, encoding a CCCH tandem zinc finger RNA-binding protein, results in defective hematopoiesis. Blood *114*, 2401-2410.

Stumpo, D.J., Byrd, N.A., Phillips, R.S., Ghosh, S., Maronpot, R.R., Castranio, T., Meyers, E.N., Mishina, Y., and Blackshear, P.J. (2004). Chorioallantoic fusion defects and embryonic lethality resulting from disruption of Zfp36L1, a gene encoding a CCCH tandem zinc finger protein of the Tristetraprolin family. Mol Cell Biol *24*, 6445-6455.

Suzuki, T., Tsutsumi, A., Suzuki, H., Suzuki, E., Sugihara, M., Muraki, Y., Hayashi, T., Chino, Y., Goto, D., Matsumoto, I., *et al.* (2008). Tristetraprolin (TTP) gene polymorphisms in patients with rheumatoid arthritis and healthy individuals. Mod Rheumatol *18*, 472-479.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., and Kageyama, R. (1994). Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. J Biol Chem *269*, 5150-5156.

Tamamaki, N., Nakamura, K., Okamoto, K., and Kaneko, T. (2001). Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex. Neurosci Res *41*, 51-60.

Taylor, G.A., Carballo, E., Lee, D.M., Lai, W.S., Thompson, M.J., Patel, D.D., Schenkman, D.I., Gilkeson, G.S., Broxmeyer, H.E., Haynes, B.F., *et al.* (1996). A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity *4*, 445-454.

Thumkeo, D., Shinohara, R., Watanabe, K., Takebayashi, H., Toyoda, Y., Tohyama, K., Ishizaki, T., Furuyashiki, T., and Narumiya, S. (2011). Deficiency of mDia, an actin nucleator, disrupts integrity of neuroepithelium and causes periventricular dysplasia. PLoS One *6*, e25465.

Trapnell, C., Pachter, L., and Salzberg, S.L. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics *25*, 1105-1111.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol *28*, 511-515.

Tyas, D.A., Simpson, T.I., Carr, C.B., Kleinjan, D.A., van Heyningen, V., Mason, J.O., and Price, D.J. (2006). Functional conservation of Pax6 regulatory elements in humans and mice demonstrated with a novel transgenic reporter mouse. BMC Dev Biol *6*, 21.

Ueno, M., Katayama, K., Yamauchi, H., Nakayama, H., and Doi, K. (2006). Cell cycle progression is required for nuclear migration of neural progenitor cells. Brain Res *1088*, 57-67.

Willardsen, M.I., and Link, B.A. (2011). Cell biological regulation of division fate in vertebrate neuroepithelial cells. Dev Dyn *240*, 1865-1879.

Wonders, C.P., and Anderson, S.A. (2006). The origin and specification of cortical interneurons. Nat Rev Neurosci *7*, 687-696.

Yang, X., Klein, R., Tian, X., Cheng, H.T., Kopan, R., and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. Dev Biol *269*, 81-94.

Yoon, K.J., Koo, B.K., Im, S.K., Jeong, H.W., Ghim, J., Kwon, M.C., Moon, J.S., Miyata, T., and Kong, Y.Y. (2008). Mind bomb 1-expressing intermediate progenitors generate notch signaling to maintain radial glial cells. Neuron *58*, 519-531.

Yoshida, M., Assimacopoulos, S., Jones, K.R., and Grove, E.A. (2006). Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order. Development *133*, 537-545.

Zhang, W., Wagner, B.J., Ehrenman, K., Schaefer, A.W., DeMaria, C.T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1993). Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol Cell Biol *13*, 7652-7665.

ACKNOWLEDGEMENTS

I would first like to thank Juergen Knoblich for giving me the opportunity to work in his lab, for great discussions and guidance throughout my studies. I want to thank my PhD committee members Friedrich Propst and Julius Brennecke for their helpful suggestions.

I would like to thank all lab members for their input, discussions and fantastic atmosphere in the lab. I would like to thank all "mouse" people for creating such great mammalian neurogenesis community.

My baymates Ilka and Yunli, thank you for all the fun, cookies, sharing all the reagents and great patience with the terrible mess on my bench.

I want to thank Elif and Anna for helpful comments and reading of the thesis.

I would like to thank Thomas Burkard for all the bioinformatic analysis and patience with all my questions.

I want to thank my family and friends for their constant support during my studies. Thank you Anna and Sara for all the love and energy that you give me every day.

CURRICULUM VITAE

Name: Marko Repic

Date of Birth: 7. 3. 1984

Place of Birth: Ljubljana, Slovenia

Nationality: Slovak

Address: Institute of Molecular Biotechnology IMBA

Dr. Bohr Gasse 3

1030 Vienna

Austria

Education:

2008-present: PhD in Molecular Biology at IMBA - Institute of Molecular Biotechnology, Vienna, Austria.

2006-2008: Msc in Molecular Biology at Comenius University, Bratislava, Slovakia

Master thesis: RNA interference as an approach towards understanding the role of carbonic anhydrase IX in cancer

2003-2006: Bsc in Biology at Comenius University, Bratislava, Slovakia

Bachelor thesis: Cultivation independent analysis of microbial communities in environmental samples